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The Role of the Tumour Suppressor p53 in the Elimination of Hepatitis B Virus-infected Hepatocytes

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Implications for Viral Clearance

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Declaration by the candidate

I hereby declare that this thesis is my own work and effort.

Where other sources of information have been used,

they have been indicated or acknowledged.

Signature:

Date: Heidelberg, January 2012

Summary

Chronic hepatitis B virus (HBV) infection is a major risk factor for the development of hepatocellular carcinoma (HCC) and more than 350 million people are chronically infected with HBV worldwide. The non-cytopathic hepatitis B virus is acting as a stealth virus and is, therefore, hardly detected by the innate immune system, thus contributing to chronicity. So far, it is known that infiltrating cytotoxic T cells are the main effector cells involved in the elimination of HBV-infected hepatocytes. However, the direct molecular mechanisms through which HBV-infected cells are eliminated by the immune system, as well as the signalling pathways up-regulated in infected host cells to combat the virus remain unclear. The present study was designed to investigate both the influence of the hepatocytes' p53 status on the outcome of HBV infection and *vice versa* the influence of HBV infection on p53 and p53 target gene activation in hepatocytes. In summary, hepatitis B virus infection triggers the activation of the DNA damage response leading to the stabilization and activation of the tumour suppressor p53 in an ATM/ATR-dependent manner. Activation of p53 upon HBV infection induces the up-regulation of different p53 target genes playing important roles in the extrinsic and the intrinsic apoptosis signalling pathway. In the present study the functionality of the CD95 pathway has been identified to be essential for the elimination of HBV-infected hepatocytes. The expression of proteins involved in apoptosis signalling leads to the alteration of the mitochondrial membrane potential, the subsequent release of cytochrome c and finally to apoptosis induction of HBV-infected hepatocytes. Furthermore, HBV infection triggers the induction of CD95L gene transactivation in an AP-1-dependent manner which leads to the so far unrecognized capability of virus-infected hepatocytes to secret CD95L by themselves.

Our results propose a new p53-dependent model for virus elimination in HBV-infected hepatocytes and show that p53 beyond its role as a tumour suppressor also plays an important role in viral clearance. The identification of prominent members of the apoptosis signalling pathways as crucial proteins activated upon HBV infection demonstrate an underlying mechanism for the diverse outcomes (clearance *vs.* chronicity) of an HBV infection. The significant contribution of p53 in cellular antiviral defence suggests a potential implication in the pathogenesis of HBV infection and opens new therapeutic options via stabilization of p53.

Zusammenfassung

Eine chronische Infektion mit Hepatitis B Virus (HBV) ist ein Hauptrisikofaktor für die Entstehung des Hepatozellulären Karzinoms. Als ein nicht-zytotoxisches Virus wird HBV kaum vom Immunsystem erkannt, agiert als ein sogenanntes „verstecktes" Virus und fördert dadurch die Entstehung einer chronischen Infektion. Kürzlich wurde beschrieben, dass in die Leber infiltrierende, zytotoxische T Zellen eine bedeutende Rolle in der Elimination von HBV-infizierten Hepatozyten spielen. Wie genau zytotoxische T Zellen Virus-infizierte Hepatozyten eliminieren und welche Mechanismen in infizierten Wirtszellen selbst aktiviert werden, um sich gegen die Virusinfektion zu verteidigen, ist jedoch noch nicht bekannt. In der vorliegenden Arbeit wurde deshalb die Rolle des Tumorsuppressors p53 auf den Verlauf einer HBV Infektion sowie der Einfluss von HBV auf p53 und p53-Zielgene untersucht. Die vorliegenden Ergebnisse beschreiben die essentielle Rolle eines intakten p53-Status sowie eines funktionellen CD95 Signalweges für die effektive Elimination HBV-infizierter Hepatoyzten durch das Immunsystem. Eine HBV-Infektion induziert die Aktivierung von DNA Reparatur Mechanismen, die zur ATM/ATR-abhängigen Stabilisierung von p53 führen. Die Stabilisierung von p53 resultiert in der Aktivierung verschiedener p53-Zielgene des extrinsischen und intrinsischen Apoptose Signalweges. Die Aktivierung von Apoptose Signalwegen führt zur Veränderung des mitochondrialen Membranpotentials, der Sekretion von Cytochrom c und endet in der Apoptose von HBV-infizierten Hepatozyten. Zudem konnte in der vorliegenden Arbeit gezeigt werden, dass HBV-infizierte Hepatozyten nicht nur sensitiv gegenüber CD95L-CD95-vermittelter Apoptose sind, sondern ihrerseits in der Lage sind CD95L zu sezernieren. Störungen innerhalb dieser Signalwege fördern die Entwicklung chronischer Infektionen und die Entstehung des Hepatozellulären Karzinoms.

Unsere Ergebnisse beschreiben ein neues p53-abhängiges Model der Viruselimination und zeigen, dass p53 über seine Rolle in der Tumorsuppression hinaus auch eine wichtige Rolle in der viralen Bekämpfung spielt. Zusammenfassend lässt sich sagen, dass die Identifizierung der für eine effektive Elimination HBV-infizierter Hepatozyten verantwortlichen Proteine einen grundlegenden Mechanismus, entscheidend für die unterschiedlichen Verläufe (Elimination *vs.* Chronizität) einer HBV Infektion, darstellt.

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1. Introduction

1.1 The tumour suppressor p53

p53, a short-lived transcription factor, belongs to the p53 family of proteins and is stabilized and activated in response to cellular stress, e.g. genomic instability, cytokine signalling, oncogene activation and virus infection. The activation and accumulation of p53 upon cellular stress is usually mediated by post-translational modifications (e.g. phosphorylation). p53 activation facilitates the transcription of p53 target genes and the activation of different cellular responses, among others cell cycle arrest, DNA repair, and apoptosis (c.f. figure 1.2). Therefore, the tumour suppressor p53 plays an important role in the inhibition of cancer development and beyond its role as a tumour suppressor also in viral defence.

p53 target genes possess one or more p53 specific response elements (REs), however p53-dependent transactivation is also dependent on the p53 protein level itself and on co-factors, such as apoptosis-stimulating of p53 protein 1 (ASPP1) with whom p53 interacts after severe DNA damage (Vousden and Lane 2007; Riley, Sontag et al. 2008; Menendez, Inga et al. 2009; Aylon and Oren 2011). Prominent target genes of p53 encode for proteins involved in the apoptosis signalling pathways, e.g. CD95, Puma and Bax as well as its negative regulator Mmd2 (Müller, Wilder et al. 1998; Vogelstein, Lane et al. 2000; Wang 2011). Beside its function as a transcription factor, p53 is also able to interact directly with members of the Bcl-2 family by acting as a Bcl-2-homology domain-3 (BH3)-only protein (Vousden and Lane 2007; Maclaine and Hupp 2009; Aylon and Oren 2011). Recently, it has been shown that p53 is also able to regulate miRNAs which are short non-coding RNAs binding to specific mRNAs and regulating protein levels by translation inhibition or mRNA degradation (Brosh, Shalgi et al. 2008; Menendez, Inga et al. 2009; Aylon and Oren 2011). Another mechanism of p53 as the guardian of the genome to prevent cancer development is the regulation of metabolic pathways. Metabolic alterations are common features of cancer cells caused through poor O_2 supply and increased oxidative stress. Basal or low levels of oxidative stress result in the expression of anti-oxidant genes promoting the repair of moderate damage, while higher or persistent stress levels lead to the induction of pro-oxidant genes, facilitating apoptosis induction (Vousden and Ryan 2009; Aylon and Oren 2011).

The crucial role of p53 as the guardian of the genome has been demonstrated in p53 knock-out mice. Even though p53$^{-/-}$ mice develop normally, they are predisposed to a wide variety of tumours (primarily lymphomas and sarcomas) by 6 months of age (Donehower, Harvey et al. 1992). Loss or mutation of p53 in general is associated with an increased susceptibility to cancer. In fact, up to 50% of human tumours carry a p53 mutation, demonstrating the outstanding role of p53 in tumour prevention (Polager and Ginsberg 2009; Whibley, Pharoah et al. 2009; Aylon and Oren 2011).

1.1.1 The p53 family gene structure

The other p53 family members, p63 and p73, are also clearly involved in human tumourigenesis and tumour response to therapy. They are sharing several p53 functions such as induction of cell cycle arrest and apoptosis (Maisse, Guerrieri et al. 2003; Müller, Schleithoff et al. 2006).

Due to the use of multiple promoters and alternative splicing, the p53 gene family is able to produce an array of different protein isoforms, including transactivation-competent full-length isoforms (TA-isoforms) and amino-terminally truncated (ΔN-) isoforms, acting as dominant inhibitors of the TA-isoforms (Melino, Lu et al. 2003; Müller, Schilling et al. 2005; Murray-Zmijewski, Lane et al. 2006). In addition to the transactivation domain, the p53 family proteins also contain (i) a DNA binding domain which enables these proteins to bind to their target sequences and (ii) an oligomerization domain which serves as an interaction surface upon homotetramer formation (figure 1.1). While p53$^{-/-}$ mice develop normally and show enhanced tumour susceptibility, p63$^{-/-}$ and p73$^{-/-}$ mice have severe defects in development but show less tumour susceptibility compared to p53$^{-/-}$ mice (Melino, Lu et al. 2003).
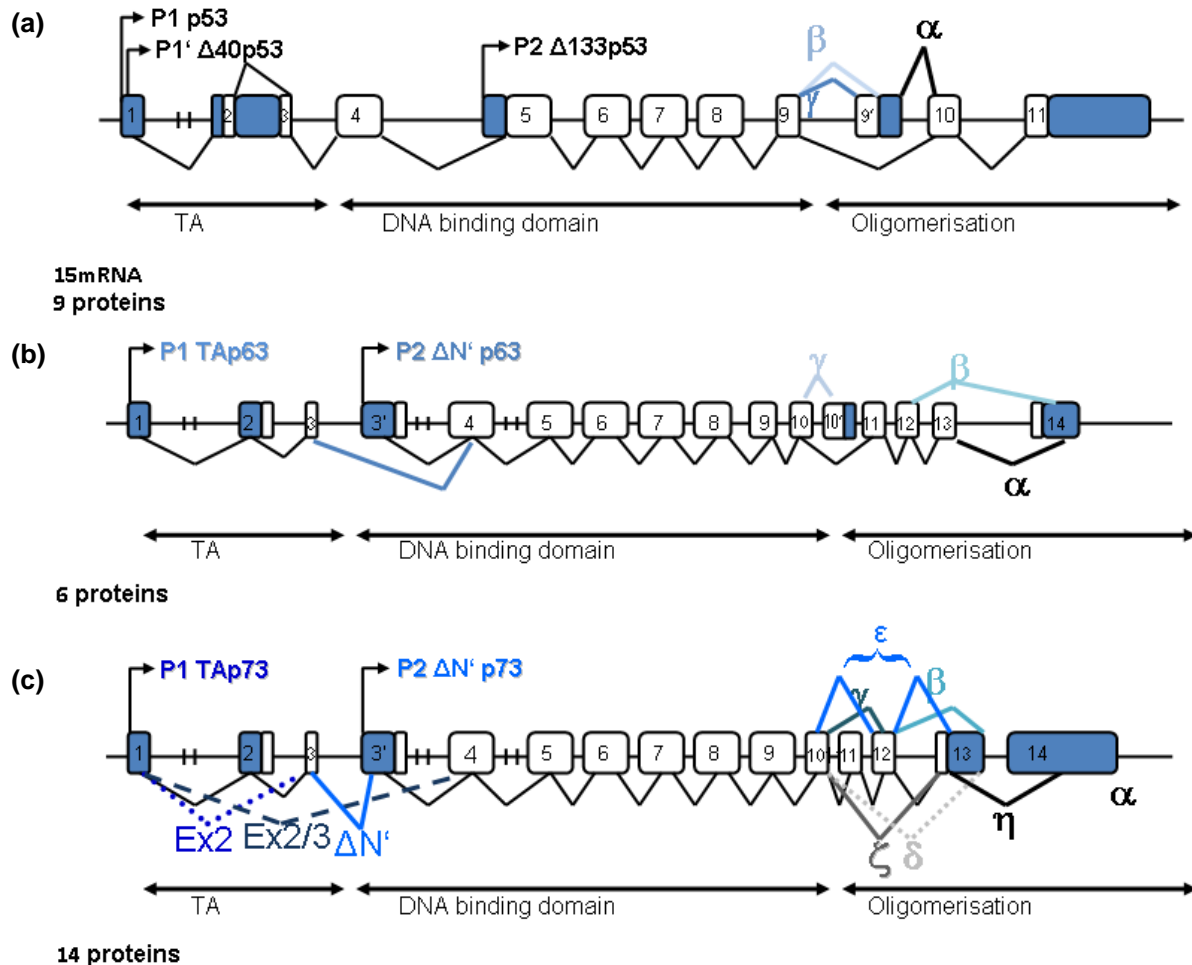
Figure 1.1 Gene structure of the p53 family (a) p53: the alternative promoters and the splicing variants are shown. The Δ40p53 protein isoforms have lost the conserved N-terminal transactivation domain. The Δ133p53 protein isoforms lack the entire transactivation domain. **(b)** p63: the alternative promoters and the splicing variants are shown. The TA-isoforms contain the transactivation domain, while ΔN-isoforms are amino-truncated proteins. **(c)** p73: the alternative promoters and the splicing variants are shown. TAp73 proteins transcribed from P1 promoter contain the conserved N-terminal transactivation domain. Ex2p73 entirely lost the transactivation domain due to alternative splicing of exons 2 and 3. ΔN-isoforms are amino-truncated proteins containing different N-terminal domains. Numbers indicate the exons encoding p53-family proteins. Figure was adapted from Müller et al. (Müller, Schleithoff et al. 2006).

1.1.2 p53 regulation and activation

p53 stabilization and activation is achieved by numerous stress sensors through phosphorylation, acetylation, ubiquitylation and methylation of specific p53 amino acid residues. More than 50 different enzymes are known to be involved in p53 modification (figure 1.2) (Maclaine and Hupp 2009; Whibley, Pharoah et al. 2009).

To prevent strong inhibitory effects on cell growth and proliferation multiple mechanisms negatively control p53 (Harris and Levine 2005; Vousden and Lane 2007). The p53 protein levels in normal cells are kept low by Mdm2, one of the most prominent p53 regulators. The E3 ubiquitin ligase Mdm2 targets p53 for ubiquitylation and subsequently proteosomal degradation. In turn, p53 positively regulates the Mdm2 gene, generating an autoregulatory negative feedback loop, which is essential for restraining p53 function (Michael and Oren 2002; Shmueli and Oren 2007; Ofir-Rosenfeld, Boggs et al. 2008; Eischen and Lozano 2009). The ability of Mdm2 to inhibit the function of p53 is blocked by e.g. ARF (alternative reading frame), another tumour suppressor gene activated upon oncogene expression or cellular stress, leading to the ubiquitylation and degradation of Mdm2 (Junttila and Evan 2009; Meek 2009; Rivas, Aaronson et al. 2010; Pang, Scott et al. 2011). Tumours not containing a p53 mutation often show an indirect p53 abrogation generated through overexpression of Mdm2 or inactivation of ARF (Polager and Ginsberg 2009; Whibley, Pharoah et al. 2009). Once activated, p53 acts as a transcription factor, activating genes involved in cell cycle arrest and apoptosis. Furthermore, p53 translocates to the mitochondria, where it physically interacts with members of the Bcl-2 family thereby stimulating apoptosis (Whibley, Pharoah et al. 2009).

1.1.3 p53 and the DNA damage response

One cellular pathway involved in p53 stabilization through phosphorylation is the DNA damage response pathway (DDR), an important barrier against tumourigenesis (Meek 2009; Wilhelm, Rufini et al. 2010) (figure 1.3). The protein kinases ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and Rad3 related), central components of the DDR, are recruited to single- and double-strand DNA breaks, thereby activated and leading to the amplification of the response by phosphorylation of several target substrates including p53 and CHK1/CHK2 (checkpoint kinase 1 and 2). Furthermore, ATM/ATR mediate the rapid destruction of Mdm2 and Mdm4, which results in the inhibition of p53 ubiquitylation. ATM/ATR-dependent phosphorylation of p53 on the key phosphorylation site serine 15 also results in the inhibition of p53 and Mdm2 interaction (Canman and Lim 1998; Canman, Lim et al. 1998; Takaoka, Hayakawa et al. 2003; Jackson and Bartek 2009; Polager and Ginsberg 2009; Bensimon, Aebersold et al. 2011; Nam and Cortez 2011).
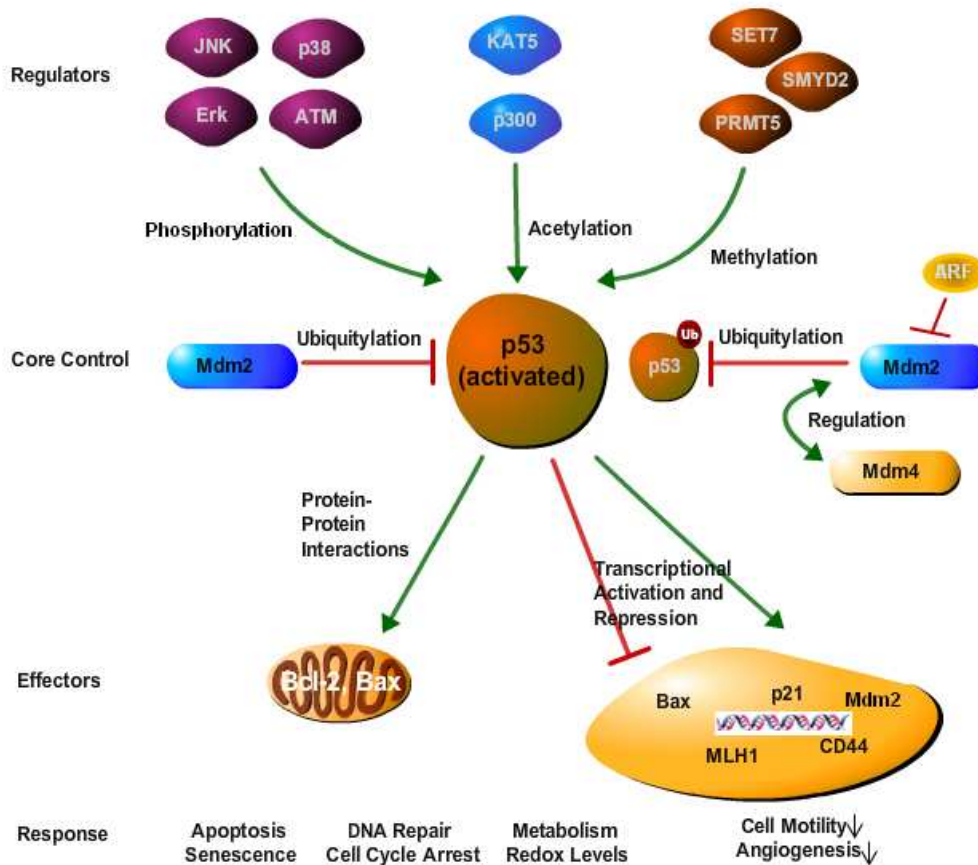
Figure 1.2 The p53 pathway The tumour suppressor p53 can be modified by more than 50 different enzymes, which alter p53 stability, cellular localization or activity. The p53 protein level in normal cells is kept low by Mdm2. Mdm2 targets p53 for ubiquitylation and proteosomal degradation. The activation of p53 by cellular and genomic stress disrupts the interaction between p53, Mdm2 and Mdm4 by posttranslational modifications. p53 can now act as a transcription factor, activating or repressing genes involved in apoptosis, cell cycle arrest and senescence. The localization of p53 at the mitochondria membrane enables a direct protein-protein interaction of p53 with members of the Bcl-2 family. ATM: ataxia-teleangiectasia mutated; Bax: Bcl-2-associated X; JNK: Jun N-terminal kinase; Kat5: K (lysine) acetyltransferase 5; MLH1: MutL protein homologue 1; PRMT5: protein arginine methyltransferase 5; SMYD2: SET and MYND domain-containing 2; Mdm2: murine double minute 2; Erk: extracellular signal-regulated kinase; p38: p38MAP kinase; p300: p300 acetyl transferase; ARF: alternative reading frame. Figure was adapted from Whibley et al. (Whibley, Pharoah et al. 2009). Pathway was generated using the pathway builder from www.proteinlounge.com.

The phosphorylation of histone H2A.X at the site of DNA damage is another very early step in the cellular response to DNA double-strand breaks. H2A.X is rapidly phosphorylated at serine 139 by ATM/ATR upon DNA damage, which causes chromatin condensation and plays an important role in efficient double-strand break repair. A further ATR/ATM down-stream target which rapidly relocates to the site of

DNA damage in a phospho-H2A.X (y-H2A.X)-dependent manner is 53BP1 (p53 binding protein 1). 53BP1 interacts with the DNA-binding domain of p53, enhances its transcriptional activity and is involved in tumour suppression (Ryan and Vousden 1998; Burma, Chen et al. 2001; Li-Weber and Krammer 2002; Wang, Matsuoka et al. 2002; Wang, Gregori et al. 2004; Gulow, Kaminski et al. 2005; Aly and Ganesan 2011).
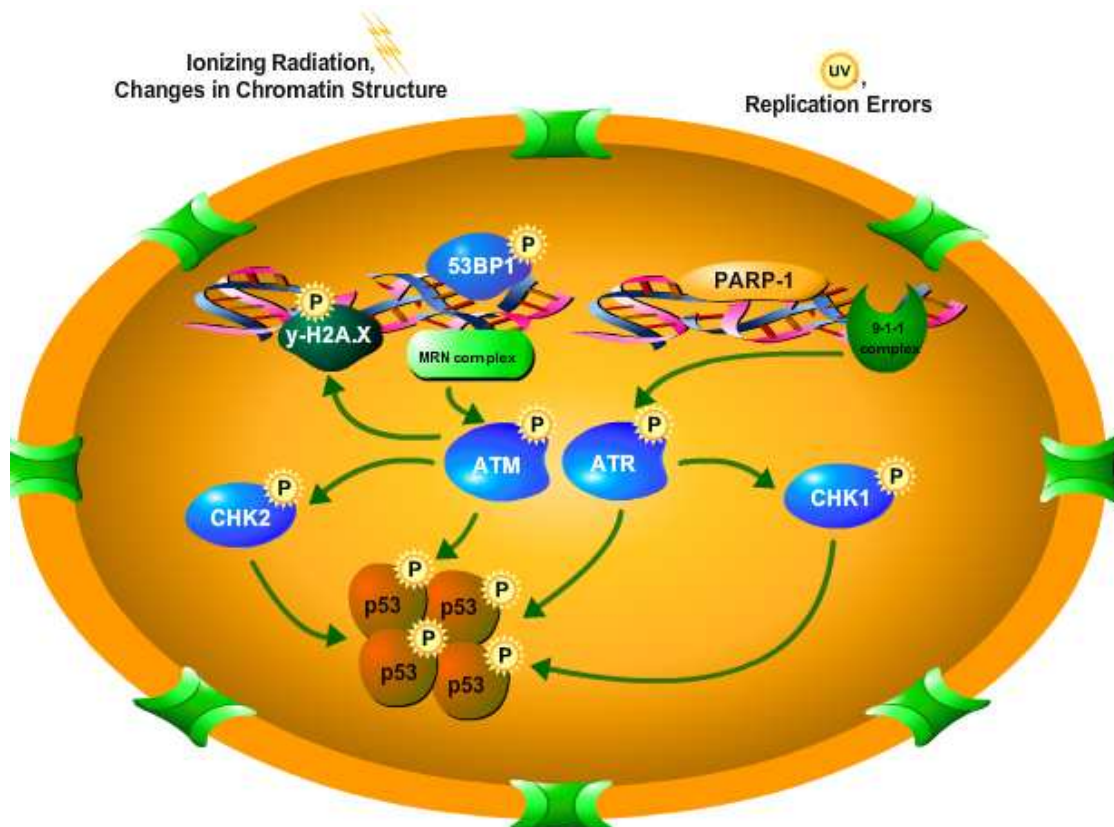
Figure 1.3 DNA damage response signalling pathway DNA double-strand breaks are recognized by the MRN (MRE11-Rad50-NBS1) complex. The MRN complex leads to the phosphorylation and activation of ATM (ataxia-teleangiectasia mutated) which in turn phosphorylates p53, CHK2 and Mdm2/Mdm4 (not shown here) as well as H2A.X. In the case of single-strand breaks the Rad3-related (ATR)-ATR-interacting protein (ATRIP) is attracted by RPA (replication protein A) (not shown here), which phosphorylates the 9-1-1 complex (Rad9, Rad1, HUS1). Ataxia-telangiectasia and Rad3 related (ATR) is activated by the 9-1-1 complex and phosphorylates p53, Mdm2 and CHK1. Another ATR/ATM down-stream target which rapidly relocates to the site of DNA damage in a phospho-H2A.X (y-H2A.X) dependent manner is 53BP1 (p53 binding protein 1). Additionally poly-(ADP-ribose) polymerase (PARP-1) swiftly binds to DNA breaks and catalyzes the addition of negatively charged PAR chains to itself and other proteins. Figure was partly adapted from Meek (Meek 2009). Pathway was generated using the pathway builder from www.proteinlounge.com.

Additionally, poly-(ADP-ribose) polymerase (PARP-1) promptly binds to DNA breaks and catalyzes the addition of negatively charged PAR chains (poly (ADP-ribose) polymers) to itself and other proteins. The binding of PARP-1 to single-strand breaks protects DNA lesions from further processing. PARP-1 plays an important role in the DNA base excision repair and is essential for the maintenance of genomic integrity. Accumulation of negatively charged PAR polymers leads to the dissociation of PARP-1 from the DNA (Schultz, Lopez et al. 2003; Jackson and Bartek 2009; Aly and Ganesan 2011). Furthermore, inactivation of PARP-1 through caspase 3-mediated cleavage is an intermediate step in programmed cell death (D'Amours, Sallmann et al. 2001). A further way of p53 phosphorylation is the Jun NH$_2$-kinase (JNK) pathway which is activated upon cellular stress and is able to control apoptosis, both positively and negatively. Phosphorylation of JNK on amino acid (aa) 183 and 185 by MKK4, which is itself phosphorylated by its upstream kinase MEKK1, leads to the increased activity of JNK. Tightly bound to its substrates e.g. p53, JNK is able to control their ubiquitinylation and phosphorylation. Under normal conditions JNK leads to the ubiquitinylation of p53, which switches to phosphorylation and stabilization of p53 under cellular stress. Moreover, the transcription factor AP-1 component c-Jun and Bcl-2 related proteins are pro-apoptotic substrates of JNK (Fuchs, Adler et al. 1998; Fuchs, Adler et al. 1998; Hamdi, Kool et al. 2005).

1.2 p53 and apoptosis

Apoptosis or programmed cell death is a highly conserved intrinsic program involved in the regulation of several physiological and pathological processes. Triggered in response to a variety of signals, two distinct but interlinked pathways, the extrinsic and intrinsic apoptosis signalling pathway, lead to the induction of apoptosis (Fulda 2009; Zuckerman, Wolyniec et al. 2009). Stimuli leading to the activation of the cell suicide machinery are among others genotoxic or cellular stress, heat shock, toxins, oncogenic transformation and bacterial or viral infection (Ashkenazi 2008). The role of p53 in apoptosis induction is well-defined. p53 is able to transactivate or repress a panel of p53 target genes playing important roles in the extrinsic and intrinsic apoptosis signalling pathway. The targets transactivated by p53 represent a large range of molecules with different functions. While p53 transactivates pro-apoptotic

members of the Bcl-2 family, the apoptosis machinery as well as several cell death receptors, it represses the transcription of anti-apoptotic genes e.g. Bcl-2 and Mcl-1. By inducing the oligomerization of the mitochondrial proteins Bax and Bak as well as by associating with anti-apoptotic proteins, p53 is able to control and influence the apoptosis machinery also in a transcription-independent manner (Chipuk and Green 2004; Chipuk, Kuwana et al. 2004; Aylon and Oren 2007; Pietsch, Sykes et al. 2008; Zuckerman, Wolyniec et al. 2009).
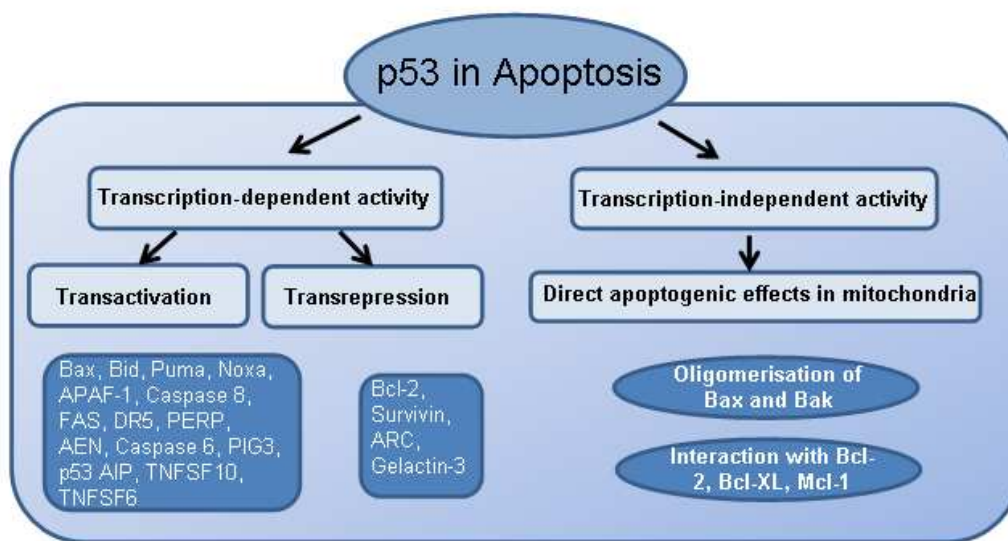
Figure 1.4 Mechanisms involved in p53-mediated apoptosis p53 induces apoptosis in a transcription-dependent and transcription-independent manner. It transactivates multiple genes encoding for proteins involved in the apoptosis machinery, such as the Bcl-2 family, cell death receptors and cell death ligands (TNFSF10 and TNFSF6). Furthermore, p53 represses the transcription of genes encoding for anti-apoptotic proteins. Direct interaction of p53 with Bax and Bak has effects on the intrinsic apoptosis pathway. Figure was modified from Zuckerman et al. (Zuckerman, Wolyniec et al. 2009).

1.2.1 Extrinsic apoptosis signalling pathway

The extrinsic or death receptor apoptosis signalling pathway is normally activated by specialized immune cells, natural killer cells (NK cells) or cytotoxic T cells, producing specific pro-apoptotic ligands. The ligands belong to the cytokine tumour-necrosis factor (TNF) superfamily and bind specifically to their corresponding pro-apoptotic death receptor expressed on the surface of target cells (Ashkenazi 2008). The death receptors, such as CD95 (Fas), TRAILR1, TRAILR2 and TNFR, belong to the tumour-necrosis factor receptor (TNF-R) superfamily (Rufini and Melino 2011). Ligation of the death receptors with their corresponding ligands is followed by the

formation of the death-inducing signalling complex (DISC) through homologous domain interactions, the recruitment of the adaptor protein Fas-associated death domain (FADD) and the activation of caspase 8. Activated caspase 8 then cleaves and activates caspase 3, 6 and 7 which induce the release of death substrates and finally cell death (Bouillet and O'Reilly 2009). Alternatively, caspase 8 can also trigger the cleavage of Bid, which translocates to the mitochondria upon cleavage and triggers cytochrome c release through the activation of Bax and Bak (Ashkenazi 2008; Fulda 2009).

One member of the TNF-R superfamily, CD95, is constitutively expressed in a wide range of tissues including the liver, which is highly sensitive to CD95-mediated apoptosis (Galle, Hofmann et al. 1995; Krammer 2000; Schilling, Schleithoff et al. 2009). Müller et al. have previously shown that the CD95 gene can be transactivated directly by binding of wild-type p53 (wt p53) to a strong p53-responsive element located within the first intron of the gene cooperating with sequences in the CD95 promoter to achieve maximal transactivation by wt p53 (Müller, Wilder et al. 1998; Schilling, Schleithoff et al. 2009).

1.2.2 Intrinsic apoptosis signalling pathway

The activation of the intrinsic apoptosis signalling pathway is regulated by members of the Bcl-2 family which can have either pro- or anti-apoptotic functions. Proteins with only a single Bcl-2 homology 3 (BH) domain are called BH3-only proteins and have pro-apoptotic features. While Bid, Bim, Puma and Noxa belong to the BH3-only protein group, the pro-apoptotic Bcl-2 family proteins Bax, Bak, and Bok possess multiple BH domains. The anti-apoptotic members of the Bcl-2 family, Bcl-2, Bcl-w, Bcl-X_L and Mcl-1 inhibit the function of the pro-apoptotic family members and are required for cell survival. Similar to CD95, the Bax gene can be transactivated directly by binding of wt p53 to one of the four p53-responsive elements located within the promoter (Miyashita and Reed 1995; Coultas and Strasser 2003; Ashkenazi 2008; Rufini and Melino 2011).

Bcl-2 family proteins regulate caspase activation through regulation of the mitochondrial outer membrane permeabilization (MOMP). Upon cell death stimuli Bax and/or Bak, present in a normal cell as inactive monomers, are activated by BH3-only proteins, e.g. Puma and Noxa, and oligomerize within the mitochondrial outer membrane. The activation of Bax and Bak results in the formation of a channel

in the mitochondrial outer membrane, permitting the release of cytochrome c (Autret and Martin 2009).
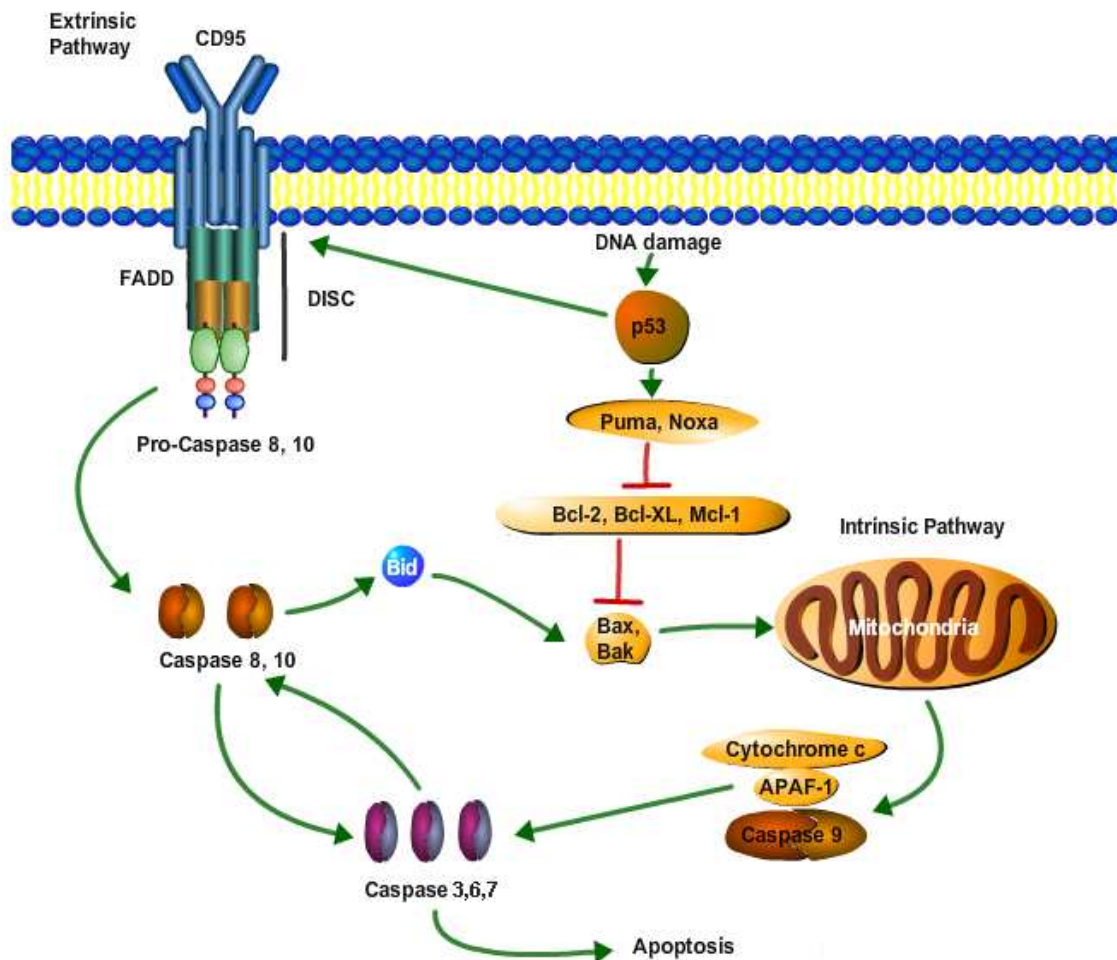
Figure 1.5 Key steps in apoptosis signalling pathways Upon cellular stress the tumour-suppressor p53 is activated. p53 in turn induces the initiation of the intrinsic apoptosis signalling pathway by up-regulating the BH3-only proteins Puma and Noxa, which subsequently activate Bax and Bak. Due to the permeabilization of the outer mitochondrial membrane, cytochrome c is released, binds to the adaptor Apaf-1 and recruits procaspase 9 into a signalling complex, the apoptosome. The extrinsic pathway is usually activated by cytotoxic immune cells, producing pro-apoptotic ligands, such as CD95L or TRAIL. Binding of the ligand to its corresponding receptor is resulting in receptor clustering and the recruitment of FADD as well as the initiator caspases 8 and 10, which form the DISC. The DISC initiates the activation of the apical caspases, driving their autocatalytic processing and their release into the cytoplasm, where they activate the effector caspases 3, 6 and 7. Both pathways can function separately, however they often interact. Initiation of the intrinsic pathway is mainly induced by p53, which also up-regulates some of the pro-apoptotic receptors. The activation of caspase 8 leads to the processing of Bid, a protein connecting the extrinsic and the intrinsic pathway. Truncated Bid stimulates Bax and Bak to activate the intrinsic pathway. Signalling pathway was partly adapted from Ashkenazi (Ashkenazi 2008). Pathway was generated using the pathway builder from www.proteinlounge.com.

Cytochrome c release is resulting in the formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome, the subsequent activation of caspase-3 and the release of death substrates (Chinnaiyan 1999; Kroemer, Galluzzi et al. 2007). As mentioned above the intrinsic apoptosis signalling pathway can also be activated by caspase 8-induced cleavage of Bid and the translocation of truncated Bid to the mitochondria (Adams and Cory 2007).

1.3 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most frequent solid tumours worldwide (Di Bisceglie 2009). It is the fifth most common cancer in men and the seventh in women, the most common primary malignant liver cancer and the third leading cause of cancer-related death in the world. With over 85% developing countries show the highest disease incidence. The incidence rate of HCC is almost equivalent to the mortality rate due to its poor prognosis (Parkin 2001; Seitz, Schleithoff et al. 2010; El-Serag 2011).
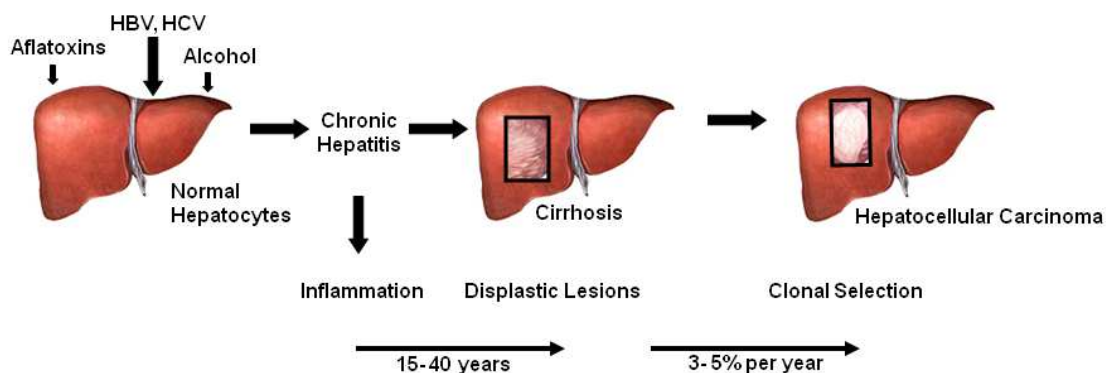
Figure 1.6 Development of hepatocellular carcinoma Chronic HBV, chronic HCV, alcohol, metabolic liver diseases, aflatoxins and associated liver cirrhosis represent major risk factors for the development of HCC. The development of HCC is a multistep process going on for several years. It includes the progressive accumulation of different genetic alterations leading to malignant transformation, occurring due to increased liver cell turnover induced by chronic inflammation, followed by subsequent injury and regeneration. At least four different signalling pathways regulating either cell proliferation or cell death are affected in HCC, i.e. pRb1 pathway, TGF-ß1 pathway, p53 family and Wnt signalling pathway. Figure was adapted from Levrero et al. (Levrero 2006) .

The p53 pathway is affected at multiple levels in hepatocellular carcinoma leading to apoptosis resistance (Wang, Hussain et al. 2002; Hussain, Schwank et al. 2007). Resistance of apoptosis is a key biological feature that contributes to the malignant phenotype of HCC (Buendia 2000; Hanahan and Weinberg 2011). More than 50% of HCCs induced by the human liver carcinogen aflatoxin (Liu and Wu 2010) and 20-40% of aflatoxin-independent HCCs in humans show mutations in p53. Micro-deletions of p14ARF occur in up to 20% of HCCs, while increased Mdm2 and gankyrin expression has been observed in the majority of HCCs. Gankyrin inhibits both the pRb- (retinoblastoma protein) and the p53-checkpoint function (Volkmann, Hofmann et al. 1994; Laurent-Puig, Legoix et al. 2001). The role of p53 and the p53 family members, p63 and p73, in hepatocarcinogenesis, therapeutic response and prognosis of HCC has been described previously (Müller, Wilder et al. 1998; Eichhorst, Müller et al. 2000; Gressner, Schilling et al. 2005; Müller, Schleithoff et al. 2006).

1.4. Hepatitis B virus

One major risk factor for the development of HCC is chronic infection with hepatitis B virus (HBV) (Kremsdorf, Soussan et al. 2006; Oyagbemi, Azeez et al. 2010). More than 350 million people worldwide are chronically infected with HBV, known to induce acute and chronic necroinflammatory liver injury and to promote hepatocarcinogenesis (Liang, Liu et al. 2007; Di Bisceglie 2009; Seitz, Schleithoff et al. 2010; El-Serag 2011). In fact, chronic HBV infection causes liver cirrhosis leading to over 50% of all HCC cases worldwide (Nguyen, Garcia et al. 2009; Grimm, Thimme et al. 2011). Every year about one million people die of HBV-related cirrhosis or HCC (Elgouhari, Abu-Rajab Tamimi et al. 2008). Integration of HBV-DNA sequences into the host genome and subsequent chromosomal rearrangements are found in approximately 90% of the patients (Ozturk 1999; Brechot, Gozuacik et al. 2000).

1.4.1 Structure and molecular virology

The hepatitis B Virus is a small non-cytopathic, parenterally transmitted, enveloped hepadnaviridae virus with circular, partially double-stranded DNA and represents the medically important prototype of enveloped DNA viruses (Schulze, Gripon et al. 2007).

The longer, negative DNA strand is the coding strand for viral mRNA and viral pregenomic RNA transcription. The shorter, plus strand is variable in length and includes two direct repeats (DR1 and DR2) at its 5' end, required for strand-specific DNA synthesis during replication. At the 5' end of both strands a short cohesive overlap region stabilizes the circular structure of the genome. Liver-specific expression of viral gene products is conferred by two enhancer elements, En1 and En2 (Chisari, Ferrari et al. 1989; Rehermann 2007; Liang 2009). The HBV genome is approximately 3200 bp in length and contains four overlapping open reading frames (ORFs). Each ORF encodes for specific structural proteins: The S gene, which can be structurally and functionally divided into the pre-S1/L-, pre-S2/M- and Surface/S-region, encodes for the viral surface envelope proteins, HBsAg. Three in-frame start codons within the ORF are responsible for production of the different surface proteins. The L- and the M-protein share the aminoacids (aa) of the S-domain and are extended by 55 (M-protein) or 108/119 (L-protein) aminoacids, called pre-S2 or pre-S1 (Schulze, Gripon et al. 2007). The precore/core gene encodes for the nucleocapsid protein HBcAg and the secreted, non-structural precore protein, HBeAg. Multiple in-frame translation initiation sites in both the S and the C gene can give rise to related but functionally distinct proteins. The polymerase ORF encodes for the viral polymerase, which is functionally divided into three domains: the terminal protein domain involved in encapsidation and initiation of minus-strand synthesis, the reverse transcriptase domain responsible for genome synthesis and the ribonuclease H (RNase H) domain which degrades pregenomic RNA and initiates replication. The fourth ORF encodes for HBxAg, a protein with multiple functions. HBx is known to be involved in signal transduction, transcriptional activation, DNA repair and protein degradation inhibition. Furthermore, it is known that HBx is required for viral replication as well as productive HBV propagation (Elgouhari, Abu-Rajab Tamimi et al. 2008; Liang 2009; Grimm, Thimme et al. 2011; Kew 2011) (figure 1.7).

Today eight HBV genotypes (A-H) – going along with differences in clinical outcome – are known. The genotypes have different geographic distributions. While

the genotypes A (serotype adw) and D (serotype ayw) are most common in the US and Europe, genotype B (serotype adw) and C (serotype adr) are more prevalent in Southeast Asia and China (Chu and Lok 2002; Dienstag 2008; Grimm, Thimme et al. 2011).
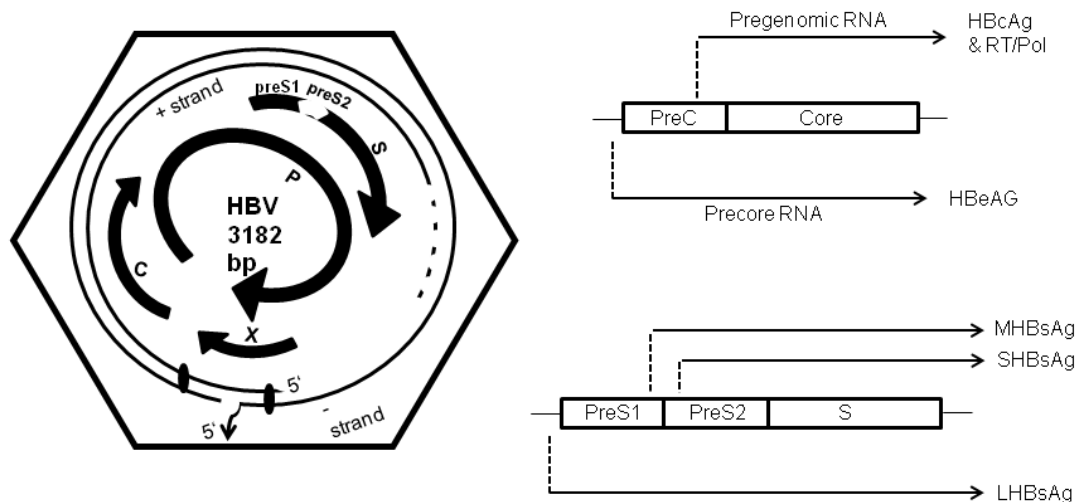
Figure 1.7 The HBV genome Genomic organization, RNA transcripts and gene products are shown on the left. HBV is a partially double-stranded enveloped DNA virus with four open reading frames, encoding for polypeptides of the surface antigen (S), the core protein (C), the polymerase (P) and the HBx protein (X). The transcription start sites of various HBV transcripts and their proteins are shown on the right. Figure was adapted from Liang et al. (Liang 2009).

1.4.2 Life cycle

The HBV life cycle has been studied in great detail; however, the hepatocyte-specific preS1 receptor, responsible for specific and probably irreversible binding of the virus to a hepatocyte remains unknown. Due to the strong liver tropism of HBV, replication exclusively takes place in hepatocytes.

The initial phase of HBV infection is the reversible and non-cell-type specific attachment to cell-associated heparan sulfate proteoglycans, followed by the irreversible binding of the pre-S1 domain in the L-protein to an so far unknown preS1-receptor (Schulze, Gripon et al. 2007; Urban, Schulze et al. 2010).

Two different virus entry pathways have been proposed: (i) endocytosis followed by the release of nucleocapsids from endocytic vesicles; (ii) fusion of the viral envelope with the plasma membrane. The direct mechanism has not been elucidated so far. The released nucleocapsid is transported to the nucleus, more precisely to the nuclear core complex along microtubules (Ganem and Prince 2004; Urban, Schulze

et al. 2010; Grimm, Thimme et al. 2011). After the release of the partially double-stranded, relaxed circular viral DNA (rcDNA) into the nucleoplasm, the plus strand of the rcDNA is completed by the viral polymerase. Viral polymerase and RNA primers used for plus-strand synthesis are finally removed by cellular enzymes. The formation of the cccDNA (covalently closed circular DNA) is achieved by covalent ligation of both strands. The cccDNA molecule is organized as a chromatin-like structure (minichromosome) and seems to be regulated by host specific factors, however, it is not amplified during mitosis but has the potential to persist in host cells for several years (Levrero, Pollicino et al. 2009; Urban, Schulze et al. 2010). The host cellular transcription machinery is used by the cccDNA to produce viral RNAs necessary for viral protein production and viral replication. This process is regulated by both cellular and viral proteins, which may modulate viral gene expression by interacting with the promoters of the four viral ORFs. All four major mRNAs use a single common polyadenylation signal, however, processing of viral RNAs, nuclear export as well as stabilization seem to be exclusively mediated by host factors (Werle-Lapostolle, Bowden et al. 2004; Urban, Schulze et al. 2010; Grimm, Thimme et al. 2011). cccDNA transcripts, transcribed by cellular RNA polymerase II, are intronless, 5'-end capped and polyadenylated. They are divided into two different species: subgenomic RNA (sgRNA) and pregenomic RNA (pgRNA), relevant for viral replication. While the pgRNA serves as a template for reverse transcription and mRNA of the viral core protein and the viral polymerase, the sgRNA directs the translation of the regulatory x protein and the three envelope proteins by free ribosomes. The three envelope proteins are directly synthesised into the membrane of the endoplasmatic reticulum (ER). The pgRNA, in contrast, forms an encapsidised complex in the cytoplasm with the core protein and the polymerase, where it is transcribed into negative-strand DNA. rcDNA is then generated by plus-strand synthesis from the negative DNA strand. The viral nucleocapsids self-assemble via complex formation of the pgRNA with the core protein and the polymerase. Nucleocapsids are either re-imported to the nucleus to generate new cccDNA or enveloped by the envelope proteins and exported via the ER and the Golgi complex. Two different groups of secreted particles are distinguished: (i) Subviral envelope particles (SVPs; 22nm) without DNA-containing nucleocapsids or (ii) Dane particles (infectious virions; 42nm) which are enveloped DNA-containing nucleocapsids.

Naturally infected hepatocytes contain up to 50 or more copies of cccDNA (Beck and Nassal 2007; Liang 2009; Urban, Schulze et al. 2010; Grimm, Thimme et al. 2011).
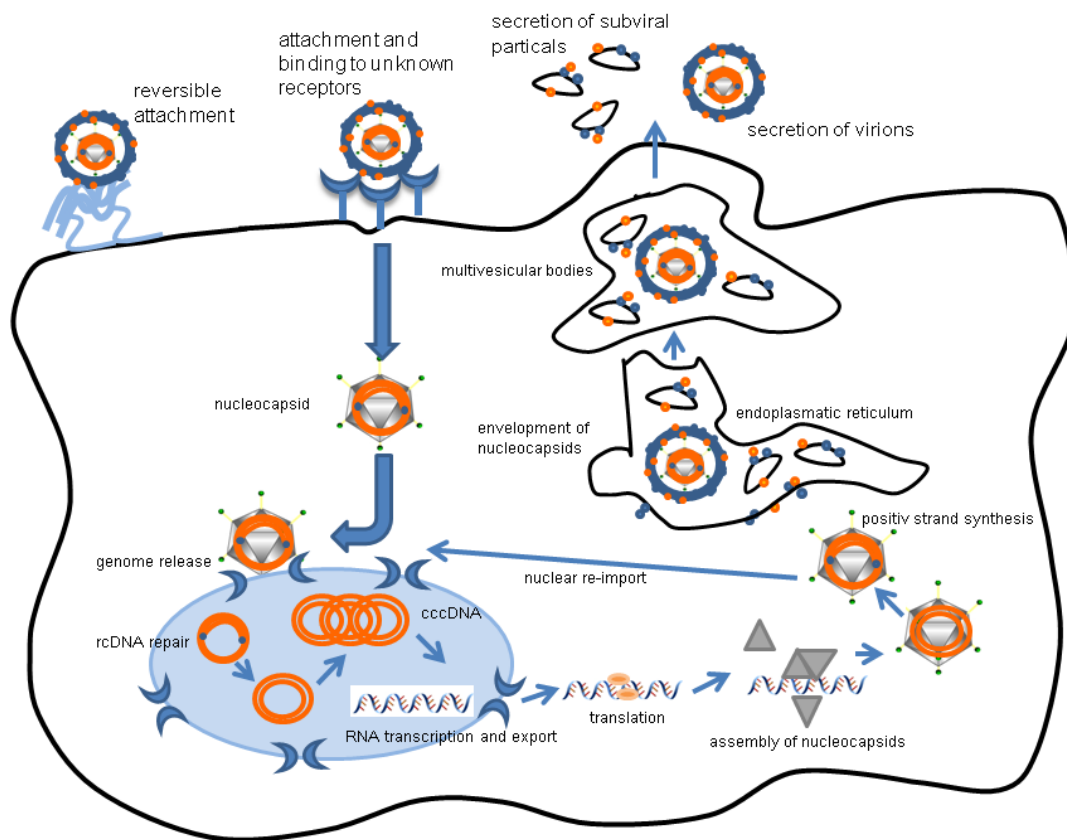
Figure 1.8 HBV life cycle Figure was adapted and modified from Urban et al. (Urban, Schulze et al. 2010) and Grimm et al. (Grimm, Thimme et al. 2011).

1.4.3 Epidemiology

HBV prevalence as well as transmission patterns vary greatly throughout the world. Being highly prevalent in Asia and sub-Saharan Africa as well as other parts of the developing countries (high-prevalence countries), western countries e.g. Western Europe and United States are so called low-prevalence countries (Elgouhari, Abu-Rajab Tamimi et al. 2008). While in high-prevalence countries HBV transmission usually occurs perinatal from chronically infected mothers, infection in low-prevalence countries predominately occurs horizontal during adolescence by sexual contacts or percutaneously by drug injection (Dienstag 2008; Grimm, Thimme et al. 2011). The risk of developing a chronic HBV infection is approximately 90% if HBV infection is acquired perinatal. Vertical perinatal HBV infection does not lead to the induction of a cellular immune response and a lifelong, persistent infection is established in most

cases (Dienstag 2008; El-Serag 2011). In contrast, up to 95% of immunocompetent adults spontaneously clear the infection (Chisari and Ferrari 1995; Lok and McMahon 2007). Even though there is a safe and effective HBV vaccine available and national HBV vaccination programs have dramatically reduced the prevalence of HBV infection, chronic HBV infection is still a major health problem (El-Serag 2011).

1.5 Hepatitis B virus infection

1.5.1 Acute and chronic HBV infection

Viral hepatitis is a necroinflammatory liver disease of variable severity. While 95% of immunocompetent adults usually suffer from a self-limiting, transient liver disease, 5% of adults and up to 90% of newborns are persistently infected with HBV. Persistent HBV infection is associated with chronic liver diseases and a high risk for the development of cirrhosis or HCC (Protzer and Schaller 2000; Guidotti and Chisari 2006; Chisari, Isogawa et al. 2010; Bauer, Sprinzl et al. 2011).

Acute HBV infection can lead to anicteric, icteric or fulminant hepatitis, however about 60% of the patients show only a mild, asymptomatic and subclinical illness, which is not even detected. Acute fulminant hepatitis occurs only in 0.5% of patients and is characterized by signs of liver failure.
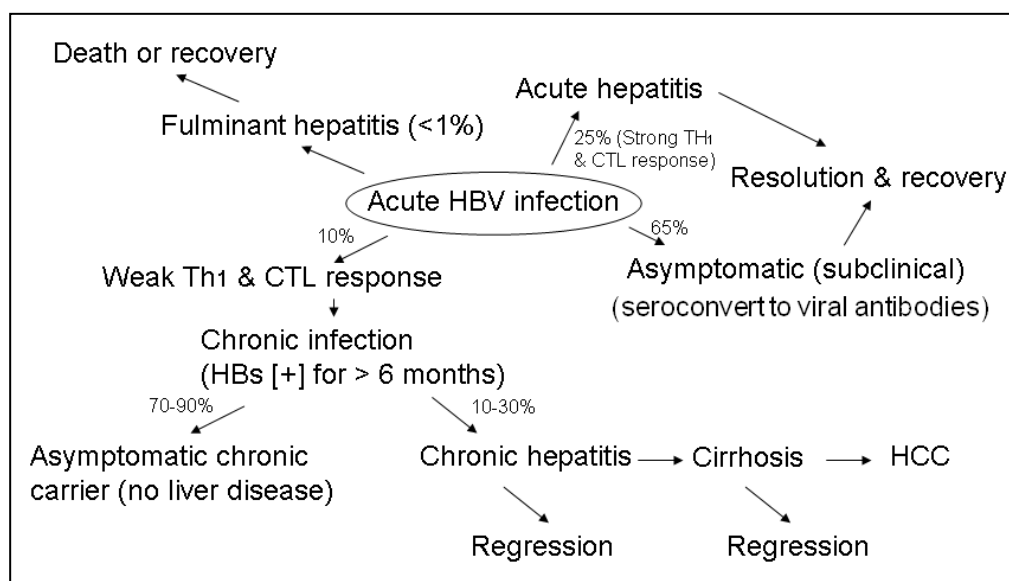
Figure 1.9 Pathogenesis and outcomes of HBV infection Figure was adapted and modified from Feitelson et al. (Feitelson and Larkin 2001).

Acute hepatitis B Virus infection is characterized by increased ALT (Alanine aminotransferase) and AST (Aspartate transaminase) levels approximately 15 weeks after infection; icteric hepatitis also shows a rise in bilirubin levels. High HBV DNA levels are not detectable during the first weeks of infection; however, HBV DNA rises together with HBeAg and HBsAg to their peak titres after 6 weeks. HBcAg-specific IgM appears earlier and persists life-long. Life-long protection is also given by the production of anti-HBs- and anti-HBe-specific antibodies. Although all clinical symptoms are resolved, small amounts of HBV cccDNA persist in a few hepatocytes and are controlled by cellular and humoral immune responses (Rehermann and Nascimbeni 2005; Elgouhari, Abu-Rajab Tamimi et al. 2008; Liang 2009).

While an acute HBV infection is characterized by a vigorous, polyclonal and multi-specific T cell response, patients with persistent, chronic HBV infection show only a narrowly focused and weak HBV-specific T cell response (Rehermann 2007; Chisari, Isogawa et al. 2010). The failure of the immune system to clear and control the infection can lead to a subsequent liver inflammation, liver cirrhosis and finally HCC as long term complications of chronic infection. In contrast to acute hepatitis, liver enzyme levels in chronic hepatitis B can be normal and no significant liver disease can be seen, especially in those patients who acquired HBV infection vertically (Chen and Chen 1999; Elgouhari, Abu-Rajab Tamimi et al. 2008; Liang 2009; Thursz, Yee et al. 2011).

1.5.2 Antiviral immune response

The immune system is a network of cells, tissues and organs working together to defend the body against infections with e.g. viruses or pathogens. However, the immune system is not only able to control most of the viruses and pathogens infecting humans, it can also provide life-long immunity after successful clearance of an infection. The innate immune system thereby functions as the first line of defence. Possessing a number of fast reacting and unspecific components, it is able to recognize and eliminate invading pathogens and viruses. The components of the innate immune system include epithelial barriers, cytokines and several cellular elements: (i) the complement system, (ii) phagocytes: granulocytes and monocytes, (iii) natural killer (NK) cells, (iv) antigen-presenting cells (APC). Pathogens are recognized by a subset of immune receptors e.g. toll-like receptors (TLRs). Phagocytosis of invading pathogens leads to the activation of the respiratory burst

and the complement system, the induction of the inflammatory response as well as cytokine/chemokine release to attract more immune cells. Antigen-presenting cells represent the antigens towards T cells and trigger the induction of the adaptive immune response.

While the innate immune system combats the invading pathogens in an unspecific manner, the adaptive immune response produces specific antibodies against a particular pathogen and protects the body against re-infection. CD4$^+$ T cells, CD8$^+$ T cells and B cells are the main cellular components of the adaptive immune response. CD4$^+$ T cells recognize peptides derived from intravesicular sources and activate macrophage- and B cell-mediated responses to the antigen. Upon activation by CD4$^+$ T cells, B cells differentiate into antigen producing cells. CD8$^+$ T cells, in contrast, recognize antigens synthesized in the cytoplasm of host cells e.g. viral proteins and peptides. They differentiate into cytotoxic T cells and are able to kill host cells infected with cytosolic pathogens. Combining the properties of the innate and the adaptive immune system, the immune system is able to control many viral infections with limited damage to host tissue (Rouse and Sehrawat 2010).

1.5.2.1 Innate immune response to viral infection

The innate immune response plays an important role in the early control of viral infections. Invading viruses are first recognized by natural neutralizing antibodies, binding to the surface of the virus and preventing viral attachment and host cell entry (Barber 2001). Viruses are also recognized by TLRs, in general TLR3, TLR7, TLR8 and TLR9 (Preiss, Thompson et al. 2008), leading to the production of pro-inflammatory cytokines, the attraction of cells involved in inflammation and the induction of the adaptive immune response. Furthermore, the activation of the complement system promotes phagocytosis or lysis of the invading viruses (Barber 2001; Pichlmair and Reis e Sousa 2007; Munz, Lunemann et al. 2009; Rouse and Sehrawat 2010). Virus-infected host cells are attacked by several humoral and cellular mechanisms to prevent virus spreading. Beside NK cells, large granular lymphocytes are potent effectors of the antibody-dependent cell-mediated cytotoxicity (ADCC) (Barber 2001; Pichlmair and Reis e Sousa 2007). The most prominent cytokines released upon viral infection are type I interferons. Type I interferons, multiple IFN-α isoforms, one IFN-β isoform and e.g. IFN-ε, can be produced by many different kind of host cells. Type II interferon, IFN-γ, in contrast is exclusively

produced by T cells and NK cells. IFN-induced antiviral function leads to the transactivation of different IFN-regulated genes (IRGs), the induction of apoptosis and cellular resistance to viral infection. Furthermore, NK cells as well as dendritic cells are activated, leading to the induction of the adaptive immune response (Barber 2001; Munoz-Fontela, Garcia et al. 2007; Koyama, Ishii et al. 2008)

1.5.2.2 Adaptive immune response to viral infection

The release of cytokines/chemokines and inflammatory molecules leads to clonal expansion of pathogen-specific T and B cells (Munz, Lunemann et al. 2009). Once activated, adaptive immune receptor cells contribute to tissue damage. Cytotoxic T cells (CD8$^+$ T cells) directly destroy virus-infected host cells through ligand-dependent activation of cell death receptor-mediated apoptosis. Some CD4$^+$ T cells boost a tissue-damaging inflammatory reaction, which can get chronic in the case of persistent virus infection. B cell-triggered antibody responses can also contribute to tissue damage by the activation of the complement system. To prevent itself against immune system-mediated tissue damage, the host produces a subset of anti-inflammatory molecules, e.g. IL-10 and TGF-ß controlling the pro-inflammatory reactions (Barber 2001; Rouse and Sehrawat 2010).

1.5.2.3 Liver immunity

The unique anatomical structure of the liver favours its immunological function. A high amount (about 30%) of the total, antigen-rich, blood is passing through the liver every minute and the small diameter of the sinusoids (blood vessels), boost the contact between lymphocytes and APCs and promote lymphocytes extravasation. Up to 10^{10} lymphocytes, conventional T cells, B cells and NK cells are located in a human liver. In the liver cytotoxic CD8$^+$ T cells usually outnumber CD4$^+$ T cells. Kupffer cells, representing the largest group of fixed macrophages are localized within the sinusoidal vascular space together with dendritic cells. Beside Kupffer cells and dendritic cells, liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs) as well as hepatocytes function as APCs (Limmer, Ohl et al. 2000; Winau, Hegasy et al. 2007; Crispe 2009). The liver, therefore, is selectively enriched with key components of the innate and adaptive immune system (Wick, Leithauser et al. 2002; Tu, Bozorgzadeh et al. 2007). Considered to be an immune privileged organ, the liver

favours the induction of tolerance by the secretion of IL-10 (Knolle, Schlaak et al. 1995), nitric oxide and reactive oxygen, resulting in the down-regulation of receptor-mediated antigen uptake as well as T cell activation. The limited initiation of a proper T cell response results in a greater significance of the innate immune system. Strong pathogen-specific signals activate abundant NK cells as well as Kupffer cells, which in turn secret pro-inflammatory mediators e.g. TNF-alpha, IL-12 and, therefore, overcome the state of active tolerance (Racanelli and Rehermann 2006; Crispe 2009; Yang, Chou et al. 2009).

1.5.2.4 The role of p53 and HBx in antiviral immunity

The tumour suppressor p53 is an important component of the antiviral defence mechanism (Takaoka, Hayakawa et al. 2003). Activated by type I IFNs p53 enforces the type I IFN response and induces apoptosis in virus-infected cells by the activation of classic p53 target genes (Munoz-Fontela, Macip et al. 2008; Rivas, Aaronson et al. 2010). Furthermore, several DNA viruses trigger cellular signalling cascades characteristic for a DDR, which also lead to p53 activation (Lilley, Schwartz et al. 2007).

Many viruses, including HBV, have evolved mechanisms to abrogate the function of p53. Viral oncoproteins, such as HBx, are able to block p53-dependent responses in infected cells and, therefore, prevent the induction of apoptosis. By forming a complex with p53, HBx sequesters this pro-apoptotic protein, prevents its entry in the nucleus, blocks its transcriptional transactivation functions and disrupts protein-protein interactions between p53 and other pro-apoptotic proteins of the apoptosis signalling pathways (Munoz-Fontela, Garcia et al. 2007; Kew 2011). On the other hand, it was reported that HBx displays also pro-apoptotic functions, even though no particular molecular mechanism has been indentified so far (Zhang, Chen et al. 2005). Up till now it is proposed that HBx can promote p53-mediated apoptosis by stabilizing p53 through the induction of E2F1 and ATR-mediated p53 activation (Wang, Hullinger et al. 2008). HBx is able to inhibit early responses to DNA damage, therefore, sensitizing the infected cells towards p53-mediated apoptosis (Matsuda and Ichida 2009). HBx transfection also led to a decrease in the mRNA and protein levels of the anti-apoptotic Bcl-2-family member Bcl-x_L, boosting the pro-apoptotic effect of HBx (Kew 2011). Similar observations have been made with the protease-deficient caspase homology c-Flip, a key regulator of the DISC (Kim and Seong

2003). HBx-induced inhibition of c-Flip results in the promotion of death receptor-mediated apoptosis, which has been described by Su et al. (Su and Schneider 1997). Furthermore, it has been shown recently that HBx sensitizes hepatoma cells to apoptotic stimuli by directly localizing to the mitochondria, altering the membrane potential and increasing cellular ROS (reactive oxygen species) production (Boya, Pauleau et al. 2004; Hu, Chen et al. 2011).

1.5.2.5 Immune control of HBV

Acute and chronic HBV infection is characterized by necroinflammatory lymphomononuclear cell infiltration and Kupffer cell hyperplasia, leading to a necroinflammatory liver disease (Chisari and Ferrari 1995; Rehermann and Nascimbeni 2005). Being a non-cytopathic virus, the immune response to viral antigens is said to be responsible for liver disease and viral clearance. In patients with an acute HBV infection a polyclonal, vigorous and multi-specific T cell response is detectable, whereas the T cell response in chronically infected patients is weak, narrowly focused and unable to control HBV replication (Chisari 1997; Guidotti and Chisari 2006; Phillips, Chokshi et al. 2010). The main cause of liver damage in HBV infection is the destruction of HBV-infected cells by CD8$^+$ effector T cells (Thimme, Wieland et al. 2003; Rouse and Sehrawat 2010). CD8$^+$ effector T cells are cross-primed by cytokine producing CD4$^+$ T cells, which are also responsible for the T cell dependent B cell response. While cytotoxic CD8$^+$ T cells are responsible for the clearance of HBV-infected hepatocytes, the B cell-induced humoral immune response neutralizes free viruses and prevents virus spread (Chang and Lewin 2007; Bauer, Sprinzl et al. 2011). Cytotoxic T cell-dependent apoptosis induction results in the release of non-infectious viral particles from apoptotic cells, thus limiting the infection (Arzberger, Hosel et al. 2010).

While the role of the adaptive immune response in HBV infection seems to be quite obvious, the role of the innate immune response in HBV infection is still poorly understood, since HBV infection is usually diagnosed long after the onset of the infection. The innate immune response is crucial for the early defence against viral infection and the induction of the adaptive immune response (c.f. 1.5.2.1). Most viral infections are recognized by pattern recognition receptors and are characterized by the release of antiviral cytokines, e.g. IFNs (Bauer, Sprinzl et al. 2011). HBV replication can be inhibited by type I INFs, but after HBV infection no IFN response

can be observed, leading to the hypothesis that HBV is acting as a "stealth" virus (Chisari, Isogawa et al. 2010).

As a "stealth" virus, HBV does not modulate host cellular gene transcription and does not induce innate antiviral immune responses in the liver. However, low levels of antiviral cytokines could be detected in a subset of patients, leading to the conclusion that HBV can be detected in some way by the host during early infection and subsequently leads to the activation of the immune response (Ait-Goughoulte, Lucifora et al. 2010).
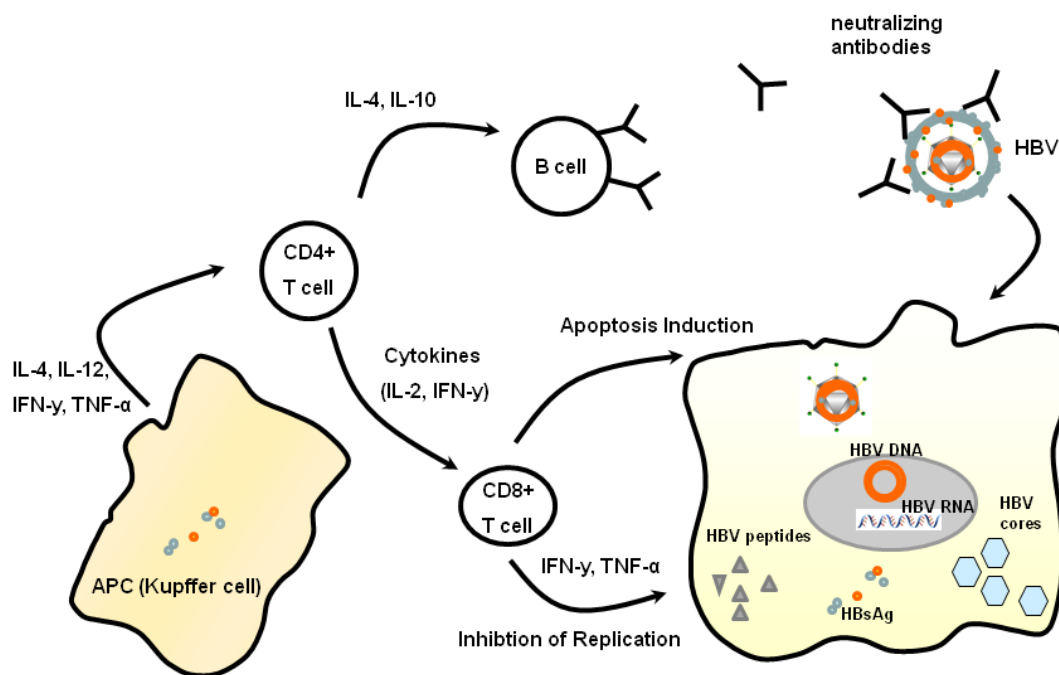
Figure 1.10 Cellular Immune Response to HBV HBV replication results in the secretion of HBsAg and virions, which are recognized and taken up by APCs. After degradation of the viral proteins, the peptides are bound to and represented on the surface of APCs via major histocompatibility complex (MHC) class I or class II molecules. The peptides are recognized by CD4$^+$ and CD8$^+$ T cells. While CD4$^+$ T cells activate antibody producing B cells and therefore the humoral immunity, CD8$^+$ T cells, with the help of CD4$^+$ T cells, are able to recognize viral peptides presented by MHC class I molecules on infected hepatocytes. Recognition of viral antigens can either lead to the inhibition of viral replication due to secretion of IFN-y and TNF-α, or more importantly to the direct induction of apoptosis of the infected hepatocytes. Figure was adapted and modified from Ganem et al. (Ganem and Prince 2004).

1.6 Aim

Chronic hepatitis B virus infection is a major risk factor for the development of hepatocellular carcinoma (HCC). With over 350 million people chronically infected with HBV worldwide this is a major health burden even though there is an effective vaccine available. Although it is well known that cytotoxic T cells play a major role in the elimination of HBV-infected hepatocytes and that apoptosis of HBV-infected hepatocytes is mediated by several molecular pathways, the direct molecular mechanisms activated in HBV-infected cells to promote virus elimination are still poorly understood. So far, the virus regulatory protein HBx has been described to play an important role in apoptosis induction by interfering with cellular proteins, that are involved in p53-dependent and p53-independent apoptosis signalling (Chirillo, Pagano et al. 1997; Wang, Hullinger et al. 2008; Arzberger, Hosel et al. 2010).

The major focus of the present study was to investigate both the influence of the p53 status of hepatocytes on the outcome of HBV infection and *vice versa*, the influence of HBV infection and the different viral proteins on p53 activation in hepatocytes. The application of gene transactivation assays, real time PCR and protein analysis aimed at the identification of potential target genes and proteins involved in the elimination of HBV-infected hepatocytes. The objective was to elucidate factors leading to the persistence of HBV and, thus, to HBV-induced hepatocarcinogenesis with focus on proteins of the extrinsic and intrinsic apoptosis signalling pathway system as an underlying mechanism for the diverse outcomes of an HBV (viral clearance *vs*. chronicity). In a second step it was investigated whether HBV-infected hepatocytes are able to secret CD95L in a similar manner as it was previously described for hepatocytes treated with chemotherapeutics. Thus, different CD95L-luc reporter constructs were used to analyse CD95L transactivation in HBV-infected hepatocytes and to identify the molecular players responsible for the activation of CD95L gene transactivation.

2. Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

The following cell lines were used:

HepG2 (human hepatoblastoma, wild-type (wt) p53) (Aden, Fogel et al. 1979), Hep3B cells (human liver carcinoma, deficient in p53 (Knowles, Howe et al. 1980), HEK293 cells (Graham, Smiley et al. 1977) and primary human hepatocytes (PHH) (Schulze-Bergkamen, Untergasser et al. 2003).

All cell lines and PHHs were cultured at 37℃ and 5% CO_2 atmosphere. Cells (except PHHs) were splitted at a ratio of 1:5 when they were 90-100% confluent.

2.1.2 Primary human hepatocytes (PHH)

All experiments with human liver tissue have been approved by the local ethics commission (University of Heidelberg/ file number 110/2000).

2.1.2.1 Isolation of PHH

PHHs were isolated by a standard two-step collagenase perfusion protocol and cultured in Williams Medium E (Maintainace Medium, Invitrogen) in collagen-coated cell culture dishes (Schulze-Bergkamen, Untergasser et al. 2003). Healthy liver tissue used for perfusion was canulated at a large branch of a vene. The liver was perfused with 500 ml Perfusion Solution I (37℃) with a flow rate of 15 ml/min to 50 ml/min. In a second perfusion step the resectate was perfused with 250 ml of collagenase containing Perfusion Solution II (37℃). Upon successful perfusion, the colour of the resectate changed from red to brown and finally beige. The perfusion was stopped as soon as the resectate softened. The liver was cut into small pieces and the cells were scratched of with a scalpel. The cell suspension was filtered through a 70 μm cell strainer and centrifuged for 5 min at 400 rpm at 10℃ without brake. The supernatant was discarded and the pellets re-suspended in 40 ml Wash Medium (4℃). This step was repeated twice, before the cells were re-suspended in PHH Medium (37℃). After the determination of cell viability and cell number, the cells were seeded in 10% FCS containing PHH Medium and maintained at 37℃,

5% CO_2. After 24 h the cells were kept in 5% FCS containing medium. From day 2 on the cells were cultured in PHH Medium without FCS.

2.1.3 Culture media and buffers

Table 2.1 Overview of cell culture media and supplements

Medium	Supplements	Cell type
Minimal Essential Medium (MEM)[¯] 500 ml	50 ml FCS[¯]; 5 ml Penicillin-Streptomycin (5000 U/ml)[¯]	Hep3B
Dulbecco`s MEM (DMEM)[¯] 500 ml	50 ml FCS; 5 ml Penicillin-Streptomycin (5000 U/ml)	HepG2
Minimal Essential Medium (MEM)[¯] 500 ml	50 ml FCS; 5 ml Hepes (1 M, pH=7,4)[¯]; 5 ml Gentamycin (10 mg/ml)[¯]	HEK293
Dulbecco`s MEM (DMEM)[¯] 500 ml	50 ml FCS; 5 ml Hepes (1 M, pH=7,4); 5 ml Gentamycin (10 mg/ml)	Jurkat 16
Williams E Medium (Maintainace Medium) 500 ml	50 ml FCS; 5 ml L-Glutamine (200 mM)[¯] 5 ml Non essential amino acids (10 mM)[¯]	PHH
Perfusion solution I (HBSS, Ca^{2+}/Mg^{2+} free) 500 ml	2.5 ml EGTA (100 nM)[±]; 1 ml Heparin[±]	PHH
Perfusion solution II 250 ml Williams E Medium	0.9 ml $CaCl_2$ (1 M)[±]; 2.5 ml Gentamycin (10 mg/ml); 200 mg Collagenase type IV (450 U/ml)[±]	PHH
Wash Medium 500 ml Williams E Medium	5.6 ml L-Glutamine (200 mM)*; 6 ml Glucose (5%)*; 11.5 ml Hepes (1 M, pH=7,4)*; 5.6 ml Penicillin-Streptomycin (5000 U/ml)* * Solutions were mixed and stored as premix at -20℃	PHH
PHH Medium 500 ml Wash Medium	5 ml Gentamycin (10 mg/ml); 0.5 ml Hydrocortison (4,85 mg/ml)[±]; 1.4 ml Insulin (280 µg/ml)[×]; 8.7 ml DMSO (78.13 g/mol)[ʳ]	PHH

[¯] Invitrogen, Karlsruhe, Germany

[ʳ] Roth, Karlsruhe, Germany

[±] Sigma-Aldrich, Deisenhofen, Germany

[×] Serva Feinbiochemica, Heidelberg, Germany

2.1.4 Calculation of cell numbers

A Neubauer hemacytometer was used to determine the cell number of homogenous cell suspensions. Cells in 2 large squares of the hemacytometer were counted and cell number was calculated according to the following formula:

(total cell number * dilution factor * 10^4)/ 2 = number of cells/ ml)

Prior counting the cell suspension was diluted 1:1 with trypan blue. Since trypan blue exclusively stains dead cells, the number of viable cells could easily be determined by light microscopy.

2.1.5 Seeding of hepatoma cell lines and PHH

All cell lines and PHHs were cultured in culture flask and seeded in cell culture multi-well plates obtained from Greiner Bio-One (CELLSTAR).

Table 2.2 Number of cells seeded in the respective culture plate

Cell number	HepG2		Hep3B		PHH	
Culture time	48h-96h	12h-48h	48h-96h	12h-48h	48h-96h	12h-48h
24 well plate	62,500	120,000	31,250	56,250	62,500	120,000
12 well plate	112,500	170,000	56,250	85,000	112,500	170,000
6 well plate	300,000	450,000	150,000	225,000	300,000	450,000
175 cm^2 cell culture flask	5,600,000	7,600,000	2,800,000	3,800,000	5,600,000	7,600,000
75 cm^2 cell culture flask	2,400,000	3,150,000	1,200,000	1,575,000	2,400,000	3,150,000

2.1.6 Spheroid culture

Multicellular tumour spheroids (MCTS) are a well-established 3-D *in vitro* system simulating the pathophysiological *in vivo* situation in tumour micro-regions. In particular, spheroids can be used as an *in vitro* model to study tumour-immune cell interactions (Gottfried, Kunz-Schughart et al. 2006). Spheroids can be generated from an array of different cell types, furthermore, it is possible to generate mixed spheroids containing two or more different cell types (Santini and Rainaldi 1999). The spheroid culture was used to further investigate and characterize the CD95-mediated apoptosis of HBV-infected hepatocytes by CD95Ligand-secreting lymphocytes.

Spheroid culture was performed as previously described (Del Duca, Werbowetski et al. 2004).

Briefly, hanging drops of Hep3B cells were generated by resuspending $1*10^6$ cells in 1 ml culture medium and suspending 25 µl drops of the cell suspension on the top cover of a 10 cm cell culture dish. After six days spheroids with optimal size and shape were selected and transferred to a collagen matrix. The spheroids were embedded in 400 µl collagen (PureCol^{TM}, Inamed, USA) in a 48-well plate and after solidification the collagen matrix was overlaid with growth medium. 24 h after embedding the spheroids were infected with the different AdHBV vectors and/or Adp53 and treated with the supernatant of PMA/Ionomycin (P/I)-stimulated (100 nM PMA, 2 µM Ionomycin) Jurkat 16 cells.

Cell migration was monitored for 16 days by photographing the spheroids with an inverted Leica phase contrast microscope at time zero and in 72 h intervals. Calculation of invaded areas was done with Zeiss Axio Vision Release 4.6.3-SP1 software.

2.2 Adenoviral constructs and infection

Replication defective adenoviral vectors generated according to the method of He et al (He, Zhou et al. 1998) were used for *in vitro* HBV infection. All adenoviral HBV vectors have been kindly provided by U. Protzer, Helmholtz Zentrum Munich, Germany. The vectors either contained the complete human wt p53 cDNA (Adp53), 1.3-fold wt HBV cDNA (AdHBV wt), X$^-$ HBV cDNA (deficient in HBV HBx protein; AdHBV X-), L$^-$ HBV cDNA (deficient in the HBV large and middle surface protein; AdHBV L-) tagged with GFP, or GFP alone (AdGFP), each under the control of the cytomegalovirus immediate/early gene promoter (CMV).

Cells were seeded in cell culture plates and infected with 10 MOI (multiplicity of infection) of recombinant adenovirus at 80% confluence at day 1 post-seeding. Infection with 10 MOI virus suspension resulted in an infection rate of 80-90%. In the case of combined AdHBV and Adp53 infection 10 MOI of each virus was used and infection with the control virus AdGFP was used to obtain an equal amount of 20 MOI in each sample.
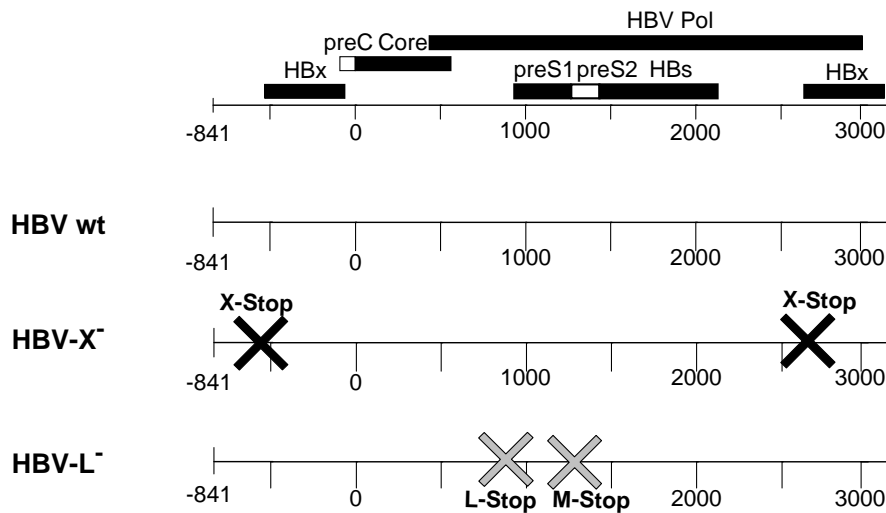
Figure 2.1 Adenoviral HBV vectors generated according to He et. al. The vectors contained the 1.3-fold HBV genome (AdHBV wt), HBx deficient 1.3-fold HBV genome (AdHBV X-) and 1.3-fold HBV genome deficient in the large and middle surface protein (AdHBV L-).

2.2.1 Production of adenoviral vectors

Pure and highly infectious AdHBV stocks were generated in HEK293 cells. The following protocol is designed for the production of 30 ml highly infectious AdHBV stocks (titre $\geq 4*10^5$ infectious virus particles/ml). $20*10^6$ HEK293 cells were seeded in 175 cm^2 cell culture flasks to be 80-90% confluent at time of infection. For the production of 30 ml virus stock six culture flasks were used. Cells were infected with 0.6 MOI of the respective adenoviral construct. 48 h - 96 h post infection (dependent on the time point the cell monolayer showed significant cytopathic features), the cells were harvested in their culture medium, transferred into 50 ml Falcon tubes, centrifuged at 1200 rpm (5 min, 4°C), resuspended in 5 ml hypotonous buffer (sterile H$_2$O with 10% PBS) and disrupted by 1 freeze/thaw cycle (-80°C O/N and 37°C water bath). After sedimentation of the cell debris (5 min; 2000 rpm; 3x), the supernatant containing infectious adenoviral HBV vectors was mixed with glycerol obtaining a final concentration of 10%.

2.2.2 Virus titration

The infectivity of the produced adenoviral HBV vector stock was estimated by the number of GFP positive Hep3B or HepG2 cells 24h post infection.
Therefore, Hep3B and HepG2 cells were seeded in a 24 well plate and infected with different MOIs of the respective virus.

Table 2.3 Amounts of virus stock solution used for titration

well number	adenoviral vector
1-3	-
4-6	2 µl
7-9	1 µl
10-12	0.5 µl
13-15	0.2 µl
16-18	0.1 µl
19-21	0.05 µl
22-24	0.02 µl

The calculation of the virus titre (infectious units (IU)/ml) is based on the following assumptions: (i) $0.56*10^5$ Hep3B cells were infected with the corresponding volumes of the virus suspension (based on the assumption that the cells proliferate over night the cell number was multiplied by 2); (ii) the minimal titre necessary to infect 100% of the seeded cells (assessed by the amount of GFP positive cells) correspond to a MOI of approximately 3 viral particles per cell. The amount of viral stock solution needed to infect about 50% of the cells, was used for the calculation of the virus titre.

$$(cell\ number*2)*(\%\ GFP\ positive\ cells*3)/\ (\mu l\ adenoviral\ vector)/100 = IU/ml$$

To estimate the functionality of the produced viral constructs, Hep3B and/or HepG2 cells were infected with 10 MOI of the respective virus. 72 h p.i. (post infection) the cells were harvested and pooled with their corresponding supernatants. To assess the amount of apoptotic cells Nicoletti staining was performed (c.f. 2.6.2).

The following apoptosis rates were expected 72 h p.i. with 10 MOI of the respective adenoviral constructs:

Table 2.4 Apoptosis rates at 72 h p.i.

adenoviral vector	HepG2	Hep3B	PHH
AdGFP	3.00-6.00%	3.00-6.00%	3.00-6.00%
Adp53	35-55%	35-55%	35-55%
AdHBV X-	10-15%	10%	10-15%
AdHBV L-	15-25%	15%	15-25%
AdHBV wt	30-35%	15%	30-35%

2.3 Plasmids

Plasmid pCMVp53wt encoding human wild-type (wt) p53 has been described previously (Ryan and Vousden 1998). Plasmid expressing mutant p53-248W was a generous gift from H. Sirma (UKE, Department of Pathology, Hamburg, Germany).

2.4 Immunoblot

2.4.1 Preparation of cell extracts

HBV-infected and untreated cells were washed with PBS and lysed in Ripa cell lysis buffer (120 mM NaCl, 1% Triton X-100, 0.5% Na. Deoxycholate, 0.1% SDS, 50 mM Tris pH=8, 1 mM DTT, 1 mM PMSF, Protease Inhibitor Mix (1 tablet/10 ml; Complete Mini, Roche, Mannheim, Germany)). Cell debris were removed by centrifugation at 13000 rpm, 4°C for 45 min. The supernatants were col lected and protein concentration was determined (BCA assay).

2.4.2 Immunoblot

Protein extracts were boiled in reducing sample buffer and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech, Freiburg, Germany) at 0.8 mA/cm² for 1.5 h using a wet blotting system with transfer buffer (25 mM Tris, 190 mM Glycin, 0.04% SDS, 20% Methanol). Protein molecular weight was controlled with the help of protein molecular weight standard (Rainbow marker, Amersham Pharmacia Biotech). Membranes were blocked with 5% non-fat dried milk powder (Roth) in PBS-T (PBS, 0.05% Tween-20) for 1 h, washed three times with PBS-T and incubated with the indicated antibodies overnight at 4°C. The membranes were further incubated with horse-radish peroxidase coupled isotype-specific secondary antibody. The secondary antibody was diluted in a range from 1:1,000 to 1:20,000 in PBS-T with 0.125% milk powder. Blots were washed three times and developed with chemiluminescence reagent (Western Lightning® Plus-ECL, PerkinElmer, Waltham, MA, USA).

The following primary antibodies have been used:

Table 2.5 Overview of primary and secondary antibodies used for immunoblotting (Secondary antibodies were purchased from Millipore (Billerica, MA, USA), Cell Signalling (Danvers, MA, USA) and Southern Biotechnology Associates (Birmingham, Alabama, USA))

1st Antibody	supplier	dilution	2nd Antibody	dilution	kDa
p53 (SC-126)	Santa Cruz Biotechnologies, CA, USA	1:500	mouse IgG2a	1:20,000	53 kDa
Phospho-ATM (Ser1981) (#9947)	Cell Signalling	1:500	mouse IgG	1:10,000	350 kDa
Phospho-ATR (Ser 428) (#9947)	Cell Signalling	1:500	rabbit IgG	1:10,000	300 kDa
Phospho-CHK1 (Ser296) (#9947)	Cell Signalling	1:500	rabbit IgG	1:10,000	56 kDa
Phospho-CHK2 (Thr68) (#9947)	Cell Signalling	1:500	rabbit IgG	1:10,000	62 kDa
Phospho-p53 (Ser15) (#9947)	Cell Signalling	1:500	mouse IgG	1:10,000	53 kDa
53BP1 (NB100-305)	Novus Biologicals, Cambridge, UK	1:1000	rabbit IgG	1:10,000	220 kDa
Bax (sc-20067)	Santa Cruz Biotechnologies	1:500	rabbit IgG	1:20,000	23 kDa
Parp (#9542)	Cell Signalling	1:1000	mouse IgG1	1:20,000	116 kDa; 89 kDa
Cyt c (556433)	Pharmingen, San Diego, CA, USA	1:500	mouse IgG2b	1:20,000	15 kDa
Bid (#2002)	Cell Signalling	1:1000	rabbit IgG	1:20,000	22kDa; 15kDa
Caspase 3 (#9661)	Becton Dickinson, Heidelberg, Germany	1:1000	rabbit IgG	1:20,000	28 kDa; 19kDa
Caspase 9 (#9502)	Becton Dickinson	1:1000	rabbit IgG	1:20,000	45 kDa; 25 kDa
CD95L (sc-7237)	Santa Cruz Biotechnologies	1:500	rabbit IgG	1:10,000	40kDa; 26kDa (cleaved)
ß-Actin (A-5441)	Sigma-Aldrich	1:1000	mouse IgG1	1:20,000	43kDa

2.5 Luminex® xMAP® technology

Principle of Luminex® xMAP® technology:

The Luminex® xMAP® technology combines the principles of flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry and is used for multiplex analysis of cell lysates. Antibodies directed against target proteins of interest are coupled with internally dyed beads, called microspheres. The addition of a specific biotinylated detection antibody results in the formation of an antibody sandwich around the target protein. Streptavidin-PE conjugate is added to complete the reaction. A panel of 100 beads, each specific for a different target molecule, allow the simultaneous detection of different proteins within one sample at the same time.

2.5.1 Quantification of protein phosphorylation

Cells were treated as described in chapter 2.2. Protein phosphorylation was quantified using the Luminex® xMAP® technology (Bio-RAD Laboratories, Munich, Germany). Cleaved PARP$^{(Asp214)}$ and total human GAPDH were purchased from Millipore, Phospho-p53$^{(Ser15)}$, p-MEK1$^{(Ser217/Ser221),}$ p-ERK1/2$^{(Thr202/Tyr204, Thr185/Tyr187)}$ and p-JNK$^{(Thr183/Tyr185)}$ were purchased from Bio-RAD Laboratories.

Cell lysis was performed 24 h, 48 h, 60 h and 72 h p.i. using the respective cell lysis buffer. Antibody capture beads, biotinylated detection antibody and Streptavidin-PE were prepared as stated in the manufacturer's protocol. The protein concentration was adjusted to 1 µg/µl; detection antibody and Streptavidin-PE were used as recommended by the manufacturer. The lysates were incubated with the capture beads overnight (4°C Millipore beads and RT Bio-RAD be ads), followed by incubation with the detection antibody (1 h, RT Millipore; 30 min, RT Bio-RAD) and the addition of Streptavidin-PE (15 min, RT). The samples treated with the Milliplex Map Kit (Millipore) were additionally treated with Amplification Buffer. Analysis was conducted using a Luminex® instrument.

2.6 Luciferase reporter assay

2.6.1 Plasmids

The CD95-luc construct containing 3.2 kb of the sequence of the CD95 gene was generated, that is, the 3'-part of the promoter, the complete exon 1 and the 5'-part of intron 1. This plasmid is denoted as p1142CD95-luc and has been engaged in all the transient transfection assays presented (Müller, Schilling et al. 2005).

The Bax-luciferase reporter plasmid (Bax-Pr/luc) (Miyashita and Reed 1995) and the respective CD95L-luciferase reporter constructs (-36/+100 CD95L-luc, -220/+100 CD95L-luc, -537/+100 CD95L-luc, -1204/+100 CD95L-luc) have been described previously (Takahashi, Tanaka et al. 1994; Li-Weber, Laur et al. 1998; Eichhorst, Müller et al. 2000; Li-Weber, Laur et al. 2000; Li-Weber and Krammer 2002; Li-Weber and Krammer 2003). (CD95L-luc plasmid cards: see Appendix)

2.6.2 Deletion of the AP-1 binding site (BS) in CD95L-luc reporter plasmids

For further analysis of the transcription factors responsible for the transactivation of the CD95L-luc reporter construct upon HBV infection it was necessary to delete the the 5' UTR AP-1 BS in the used CD95L-luc constructs.

2.6.2.1 Preparation of linerazied vectors by restriction digestion

Deletion of the AP-1 binding site in the following CD95L-luc constructs was performed using the In-Fusion® HD Cloning System CE from Clontech (Mountain View, CA, USA): -36/+100 CD95L-luc, -220/+100 CD95L-luc, -537/+100 CD95L-luc, -1204/+100 CD95L-luc. The luciferase constructs were digested with restriction enzymes (XhoI and HindIII) to generate linearized vectors and to isolate their corresponding inserts (c.f. 2.6.3.2). The vectors and their inserts were purified using QIAEX®II Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

2.6.2.2 PCR Amplification of target fragment and AP-1 BS deletion

2.6.2.2.1 PCR primer design

The following primers were designed according to the manufacturer's protocol (http://bioinfo.clontech.com/infusion). The 5' end of each primer contains 15 bases

homologous to 15 bases at the end of the vector. The designed primer induced an AP-1 BS deletion into the amplified inserts.

Table 2.6 Primer sequences for insert amplification. Primers were purchased from Eurofins mwg I operon (Ebersberg, Germany).

Forward primer
HindIII restriction site (fwd) -36: 5' CTGGATCTCGAAGCTTACTCTATAAGAGAGATCCAGCTTG 3'
HindIII restriction site (fwd) -220: 5' CTGGATCTCGAAGCTTCAGCAACTGAGGCCTTGAAGGC 3'
HindIII restriction site (fwd) -537: 5' CTGGATCTCGAAGCTTCATGGTCTCTCCCCTCAGAG 3'
HindIII restriction site (fwd) -1204: 5' CTGGATCTCGAAGCTTCGAGGGTGTCTGATATTCTGATAT 3'
Reverse Primer
XhoI restriction site (rev): 5' CGGAATGGATCTCGAGGGCCAGCCCCAGCAAACG 3'

2.6.2.2.2 PCR amplification of the CD95L gene target fragments

For amplification of the insert the Advantage® HD Polymerase Mix (Clontech) was used. Reagents were thawn on ice, the PCR reaction was assembled at RT.

Table 2.7 PCR Amplification Mix

Reagents	Volume/ final concentration
Sterile deionized H_2O	ad 25 µl
5x Advantage HD Buffer	5 µl
dNTP Mixture	2 µl
Primer 1 (fwd)	0.2 µM
Primer 2 (rev)	0.2 µM
Advantage HD Polymerase (2.5 units/µl)	0.25 µl
Template	10 ng

For insert amplification the three step PCR method was used and adapted to every insert:

Table 2.8 PCR conditions

	-36/ +100	-220/ +100	-537/ +100	-1204/ +100
98℃	00:00:10	00:00:10	00:00:10	00:00:10
55℃	00:00:05	00:00:05	00:00:05	00:00:05
72℃	00:00:07	00:00:12	00:00:30	00:01:30

2.6.2.3 In-Fusion cloning procedure

Unpurified PCR products (5 µl) were treated with 2 µl cloning enhancer and incubated at 37℃ for 15 min followed by an incubation at 80℃ for 15 min. For the Infusion cloning reaction the reagents were thawn on ice and mixed:

Table 2.9 In-Fusion Cloning Reaction

	Insert	neg. control	pos. control
5x In-Fusion HD Enzyme Premix	2 µl	2 µl	2 µl
Linearized Vector	200 ng	1 µl	1 µl (pUC19 control)
Cloning Enhancer-Treated PCR Fragment	2 µl	-	2 µl (control insert)
dH$_2$O	X µl	7 µl	5 µl
Total Volume:	**10 µl**	**10 µl**	**10 µl**

The cloning reaction was incubated at 50℃ for 15 min a nd then placed on ice.

2.6.3 Plasmid propagation

2.6.3.1 Transformation

Routine cloning was performed using the One Shot Top10 competent cells from Invitrogen (In-Fusion cloning was performed using Stellar^{TM} Competent Cells from Clontech). 50 µl *E. coli* suspension was thawn on ice. To each aliquot 10 ng/µl ligation reaction (plasmid) (routine cloning) or 5 µl In-Fusion Cloning reaction (In-Fusion cloning) was added and the tubes were further incubated on ice (30 min). Subsequently, heat shock was performed at 42℃ for 30 sec. (routine) or at 42℃ for 60 sec. (In-Fusion cloning), followed by 2 min on ice. After the addition of 250 µl (routine) or 80 µl (In-Fusion cloning) S.O.C Medium (37℃) the vials were incubated for 1 h at 37℃ with agitation at 225 rpm. 20 µl of each transformation was spread on

LB agar plates with Ampicillin (4 µg/ml). Inverted agar plates were incubated at 37℃ O/N.

2.6.3.2 Plasmid minipreparation and restriction endonuclease digestion

A single plasmid containing bacterial colony was picked from a freshly streaked agar plate and was transferred into 6 ml LB-Medium (Ampicillin 4 µg/ml) and incubated at 37℃ O/N. Bacteria were pelleted by centrifugation (5 min, 5000 rpm) and resuspended in 200 µl Flex1 Buffer (10 mM EDTA, 200 µg/ml RNase A, 100 mM Tris, pH= 7.5). 200 µl Flex2 Buffer (0.2 mM NaOH, 1% SDS) was added and the contents were gently inverted several times and incubated for 5 min at RT. After the addition of 200 µl Flex3 Buffer (3 M Potassium, 5 M Acetat, pH= 5.75) the solution was incubated on ice for 5 min and pelleted by centrifugation (5 min, 13.000 rpm, RT). The clear supernatant was transferred into a new tube. Nucleic acids were precipitated by the addition of an equal volume of isopropanol and subsequently pelleted by centrifugation. The DNA was washed with 70% ethanol, air dried for 10 min and resuspended in 15 µl H_2O.

For DNA digestion the following restriction endonuclease digestion was used:

Table 2.10 Digestion setup for CD95L-luc plasmids

10 µg DNA
10 µl Fast Digest®HindIII
10 µl Fast Digest®XhoI
5 µl 10x Buffer (green)
Ad 15 µl H_2O

Following digestion at 37℃ for 30 min, the digestion reaction was loaded on an agarose gel (1% agarose in TAE Buffer, 7 µl Ethidium-bromide). Gel running was performed for 1 h at 80 V. A 100 bp DNA marker (Fermentas, St. Leon-Rot, Germany) was used to control the size of DNA fragments.

2.6.3.3 Plasmid maxipreparation

Plasmid containing bacterial hosts were transferred into 200 ml LB-Medium (Ampicillin 4 µg/ml) and incubated at 37°C O/N. Cells were pelleted by centrifugation at 4500 g for 15 min at 4°C. The pellet was resuspende d in Resuspension Buffer RES + RNase A before cell lysis buffer was added and incubated for 5 min at RT. Meanwhile the column together with the inserted column filter was equilibrated.

After incubation the suspension was neutralized and immediately loaded on the column, emptied by gravity flow. After washing of the column with equilibration buffer and the removal of the filter, the column was washed with wash buffer and the plasmid DNA was eluted with elution buffer and collected. Plasmid DNA was precipitated with isopropanol, pelleted by centrifugation (5000 g, 15 min, 4°C), washed with 70% ethanol, pelleted and air dried for 15 min. The DNA was reconstituted in 200 µl sterile dH$_2$O and stored at 4°C (Plasmid DNA purification Kit (Nucleo Bond® Xtra Maxi), Macherey Nagel, MA, USA).

2.6.4 Transfection

Transient transfections of luciferase reporter constructs were performed using FuGENE 6 (Wang, Gregori et al. 2004). 24 h after transfection, cells were infected with 10 MOI of the respective adenovirus or 30 µg/ml bleomycin (Cell Pharm GmbH, Hannover, Germany). 48 h after infection, cells were harvested and assayed for Luciferase activity as described by the manufacturer (Promega, Mannheim, Germany).

To inhibit CD95L transactivation, HBV-infected cells were also pre-treated with a JNK II inhibitor (IC$_{50}$= 40 nM), a reversible inhibitor of the c-Jun N-terminal kinase (InSolution™ JNK Inhibitor II, Calbiochem, Darmstadt, Germany). To stimulate CD95L-luc transactivation 0.1 µg/ml recombinant human TNF-alpha (rh TNF-alpha, ImmunoTools, Friesoythe, Germany) was added. Furthermore, the TNF-receptor was blocked with 10 µg/ml Etanercept (Enbrel®) and CD95L-luc transactivation was assayed. In general, the cells were pre-treated with the inhibitors (JNK II inhibitor and Enbrel) 12 h prior infection and retreated after adenoviral infection, while TNF-alpha stimulation was only performed after infection. The generation of reactive oxygen species (ROS) was inhibited 30 min prior infection with 20 mM N-Acetyl-L-cysteine (NAC) from Sigma-Aldrich.

2.7 FACS

2.7.1 Principle of FACS measurement

Fluorescent-activated Cell Sorting (FACS) can be used to analyze and count single cells within a cell suspension. Equipped with up to four different lasers, it allows simultaneous multiparametic analysis. The forward scatter (FSC) and the sideward scatter (SSC) are used to differentiate the cells due to their size and granularity. Additional staining with antibodies coupled with a fluorescent dye to detect specific surface structures or intracellular proteins results in the emission of characteristic light signals while passing focused lasers. Emission is processed by the electronic detection apparatus.

2.7.2 Sub-G1 (Nicoletti) staining for the detection of apoptosis

Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide-stained nuclei. Apoptotic cells with degraded DNA appear in the $subG_0/G_1$ peak (Nicoletti, Migliorati et al. 1991) (FACSCalibur® flow cytometer (Becton Dickinson); CELLQuest® software).

HBV-infected hepatocytes floating in the culture medium were collected by centrifugation at 200 g. Adherent hepatocytes were harvested by incubation with 1% trypsin for 2 min. The cells were washed with PBS, suspended in hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X and 50 ng/ml propidium iodide) and incubated at 4℃ for 2 h and analysed using FACSCalibur .

2.7.3 Cell surface staining

Cell surface expression of CD95, TRAILR1 and TRAILR2 after HBV infection was assessed by FACSCalibur®. A total amount of $2*10^5$ HBV-infected hepatocytes were detached with EDTA (2 mM) treatment 72 h p.i. and stained with APO-1-1, TRAILR1 and TRAILR2 or control mAb IgG1 for 30 min at 4℃. P rimary antibody staining was followed by biotinylated secondary goat anti-mouse antibody and Streptavidin-PE incubation (20 min, 4℃). To determine the amount of apoptotic cells propidium iodide was added (1:2000).

Table 2.11 Overview of primary and secondary antibodies used for flow cytometry

1st Antibody	dilution	2nd Antibody	dilution	3rd Antibody	dilution
APO-1-1 *(Enzo Life Science)*	1:50	Biotinylated secondary goat anti-mouse antibody *(Southern Biotechnology Associates, Birmingham, Alabama, USA)*	1:200	Streptavidin–PE *(Pharmingen)*	1:200
TRAILR1 *(Alexis)*	1:100	Biotinylated secondary goat anti-mouse antibody *(Southern Biotechnology Associates)*	1:200	Streptavidin–PE *(Pharmingen)*	1:200
TRAILR2 *(Alexis)*	1:100	Biotinylated secondary goat anti-mouse antibody *(Southern Biotechnology Associates)*	1:200	Streptavidin–PE *(Pharmingen)*	1:200
control mAb IgG1 *(Santa Cruz Biotechnology)*	1:200	Biotinylated secondary goat anti-mouse antibody *(Southern Biotechnology Associates)*	1:200	Streptavidin–PE *(Pharmingen)*	1:200
control mAb IgG1 *(Santa Cruz Biotechnology)*	1:200	APC-labelled affinity purified $F(ab')_2$-fragment goat anti-mouse *(Dianova, Hamburg, Germany)*	1:200		
control anti-rabbit IgG *(Santa Cruz Biotechnology)*	1:200	APC-labelled affinity purified $F(ab')_2$-fragment goat anti-rabbit *(Dianova)*	1:200		
Bax *(Santa Cruz Biotechnology)*	1:50	APC-labelled affinity purified $F(ab')_2$-fragment goat anti-mouse *(Dianova)*	1:200		
Noxa *(Santa Cruz Biotechnology)*	1:50	APC-labelled affinity purified $F(ab')_2$-fragment goat anti-mouse *(Dianova)*	1:200		
Puma *(Santa Cruz Biotechnology)*	1:50	APC-labelled affinity purified $F(ab')_2$-fragment goat anti-rabbit *(Dianova)*	1:200		
Caspase 3 *(Cell Signalling)*	1:50	APC-labelled affinity purified $F(ab')_2$-fragment goat anti-rabbit *(Dianova)*	1:200		
Caspase 9 *(Cell Signalling)*	1:50	APC-labelled affinity purified $F(ab')_2$-fragment goat anti-rabbit *(Dianova)*	1:200		

2.7.4 Detection of intracellular proteins

Expression of intracellular proteins involved in apoptosis signalling was analyzed by intracellular fixation and staining for the respective proteins. A total amount of $2*10^5$ HBV-infected hepatocytes were detached with 1% trypsin 48 h post infection (p.i.). Cells were incubated with 100 µl Cytofix/ Cytoperm (Becton Dickinson) for 20 min (4℃, dark). Cells were stained with anti-Bax, or anti -Noxa mouse anti-human IgG1 or control IgG1 antibody, anti-Puma rabbit anti-human IgG or control anti-rabbit IgG, cleaved caspase-3 antibody or caspase-9 antibody anti-human IgG or control anti-rabbit IgG. APC-labelled affinity purified F(ab')$_2$-fragment goat anti-mouse or anti-rabbit IgG Fc antibody was used as secondary detecting reagent. Before analyzing the samples by flow cytometry, cells were washed twice with 200 µl Perm/Wash solution (Becton Dickinson) and resuspended in 50 µl Perm/Wash solution.

2.7.5 Determination of mitochondrial membrane potential

Cells were harvested, washed once with PBS and incubated with 20 nM 1,10 dihexadecyl-3,3,30,30-tetramethylin-docarbocyanine perchlorate (DiIC1), (Invitrogen) for 20 min at 37℃ (dark), washed once and analyzed at a n excitation of 633 nm by flow cytometry (Seitz, Schleithoff et al. 2010).

2.8 Quantitative real time PCR

2.8.1 Introduction

Quantitative real time PCR (qPCR) reaction is the most sensitive technique for cDNA quantifications and detections. Quantity can be either given in an absolute number of copies (absolute quantification) or as a relative amount compared to reference genes (relative quantification). In contrast to a traditional PCR, a real time PCR reaction is combined with a fluorescent detection system. The two different fluorescent detection systems available today are (i) non-specific DNA intercalating fluorescent dyes, e.g. SYBR Green and (ii) sequence-specific oligonucleotide DNA probes, labelled with a fluorescent reporter, permitting detection after hybridization with its corresponding DNA target. The probe-based detection system was used for the following experiments.

2.8.2 qPCR conditions and protocols

Total RNA was extracted from Hep3B-, HepG2-cells and PHHs cells infected with 10 MOI AdGFP or Adp53, AdHBV wt, AdHBV-X⁻, AdHBV-L⁻, using the RNeasy Mini-Kit (Qiagen). The cDNAs were synthesised from total RNA (1 µg) using the QuantiTect Reverse Transcription Kit, Qiagen.

Table 2.12a Genomic DNA elimination reaction components

	1x
gDNA Wipeout Buffer, 7x	2 µl
Template RNA	1 µg
RNase-free water	x µl
Volume	**14 µl**

Table 2.12b ReverseTranscription Master-Mix

	1x
Quantiscript® Reverse Transcriptase	1 µl
Quantiscript RT Buffer (5x, includes Mg^{2+} and dNTPs)	4 µl
RT Primer Mix (optimized blend of oligo-dT and random primers)	1 µl
Volume	**6 µl**

Table 2.13 Primer sequences and probes used for qPCR analysis

Target gene	Primer sequence		Probe number
hCD95	fwd: atggccaattctgccataag	rev: tgactgtgcagtccctagctt	#65
hCD95L	fwd: gctggcagaactccgaga	rev: ttttcaggggggtggactg	#20
hBax	fwd: atgttttctgacggcaacttc	rev: atcagttccggcaccttg	#57
hNoxa	fwd: ggagatgcctgggaagaag	rev: cctgagttgagtagcacactcg	#67
hPuma	fwd: gacctcaacgcacagtacga	rev: gagattgtacaggaccctcca	#68
hApaf-1	fwd: ggcattcctgttgtctcttctt	rev: aaacaactggcctctgtgg	#39
PBGD	fwd: cctgtttaccaaggagcttga	rev: ggagtgaacaaccaggtcca	#37
HPRT	fwd: tgaccttgatttattttgcatacc	rev: cgagcaagacgttcagtcct	#26
HMBS	fwd: tgtggtgggaaccagctc	rev: tgttgaggtttccccgaat	#73

The entire genomic DNA elimination reaction was incubated for 2 min at 42℃ and added to each tube containing Reverse Transcription Master-Mix. The RNA mix was incubated for 15 min at 42℃, inactivated for 3 min at 95℃ and stored at -20℃.

Transcript analysis was done with primer pairs purchased from Sigma-Aldrich and UPL probes purchased from Roche, Mannheim, Germany.

Endogenous reference genes were used to correct for variation in RNA content and variation in the efficiency of the reverse transcription reaction. All primers were designed using the Probe Finder qPCR assay design software (www.universalprobelibrary.com).

PCR conditions have been used as described by the manufacturer (LightCycler 480 Probes Master Kit; Roche).

Table 2.14 Reaction mix for real time PCR. *cDNA was diluted 1:50 for real time PCR (CD95L analysis 1:5)

	1x
Buffer	7.5 µl
Primer 10 µM	0.3 µl
Probe10 µM	0.15 µl
H$_2$O	3.3 µl
	11.25 µl
cDNA*	3.75 µl

Table 2.15 Running conditions LightCycler 480

	Cycles	Target (℃)	Acquisition Mode	Time	Analysis Method
Preincubation	1	95	None	00:10:00	None
Amplification	55	95	None	00:00:10	
		60	single	00:00:30	Quantification
Cool Down	1	40	None	00:00:10	None

Analysis was performed by the LightCycler 480 quantification software.

2.9 Enzyme-linked immunosorbent assay (ELISA)

For the quantitative analysis of CD95L (FasL) secretion after HBV infection enzyme-linked immunosorbent assay (ELISA) was used. The Fas Ligand ELISA Kit detecting soluble and membrane bound CD95L was purchased from Calbiochem and was performed according to the manufacturer's protocol. Briefly, the 96 well microtiter plate was captured with the biotinylated detection antibody and was incubated simultaneously at RT for 3 h with the standards and samples. After washing conjugate diluent was added and incubated for 30 min at RT. Conjugate diluent was

removed with an additional washing step and the samples were incubated with TMB substrate for 30 min at RT in the dark. After the addition of stop solution the absorbance was measured at a wavelength of 450/595 nm.

2.10 Induction of apoptosis

To induce CD95 receptor-mediated apoptosis the CD95 receptor was stimulated with the agonistic monoclonal antibody anti-APO-1 IgG3κ (kindly provided by P. Krammer, DKFZ, Heidelberg, Germany) at a concentration of 1 mg/ml as described (Chinnaiyan, Tepper et al. 1996). Furthermore, HBV-infected hepatocytes were treated with 3x concentrated (Vivaspin) supernatant of Jurkat 16 cells at 24 h post-infection. Prior to supernatant collection, Jurkat 16 cells were pretreated with PMA/ Ionomycin for 24 h at a concentration of 100 nM PMA and 2 µM Ionomycin to allow CD95L secretion. The amount of apoptotic hepatocytes was determined via Nicoletti staining 72 h after infection. APG101 (10 µg/ml), an antagonistic antibody against CD95L, was used to inhibit the CD95-CD95L dependent apoptosis.

2.11 Kits

2.10.1 RNA extraction

RNeasy Mini-Kit, Qiagen, Hilden, Germany

2.11.2 RT and qPCR

QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany
LightCycler 480 Probes Master Kit; Roche, Mannheim, Germany

2.11.3 Plasmid propagation

One Shot Top10, Invitrogen, Karlsruhe, Germany
Plasmid DNA purification Kit (Nucleo Bond® Xtra Maxi), Macherey Nagel, MA, USA

2.11.4 Cloning

In-Fusion HD Cloning Kit, Clontech, Mountain View, CA, USA
Advantage HD Polymerase, Clontech, Mountain View, CA, USA
QIAEXII Gel Extraction Kit, Qiagen, Hilden, Germany

2.11.5 Restriction enzymes

FastDigest® XhoI 5'…C^TCGAG…3' (#FD0694, Fermentas, St. Leon-Rot, Germany)

FastDigest®HindIII 5'…A^AGCTT…3' (#FD0504, Fermentas, St. Leon-Rot, Germany)

2.11.6 Phosphoplex

Milliplex® Map Kit, Millipore, Billerica, MA, USA

Bio-Plex Cell Lysis Kit, Bio-Rad, Munich, Germany

Bio-Plex Phosphoprotein and Total Target Assays, Bio-Rad, Munich, Germany

Bio-Plex Phosphoprotein Detection Reagent Kit, Bio-Rad, Munich, Germany

2.11.7 FACS analysis

Cytofix/Cytoperm, Becton Dickinson, Heidelberg, Germany

2.12 Signalling pathways

The signalling pathways were generated with the help of the pathway builder from protein lounge (www.proteinlounge.com).

2.13 Statistical analysis

All data were analyzed by ANOVA (analysis of variance) or Student's t-test to test for statistical significance. Statistical analysis was carried out using the SAS software system (SAS Institute Inc., Cary, NC, USA). p-Values ≤ 0.05 were considered statistically significant.

2.14 Chemicals

Acrylamide (30%)	Roth, Karlsruhe, Germany
Agar-Agar	Roth, Karlsruhe, Germany
Agarose	Roth, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich, Deisenhofen, Germany
APS	Roth, Karlsruhe, Germany
BES	Sigma-Aldrich, Deisenhofen, Germany
ß-Mercapto-Ethanol	Roth, Karlsruhe, Germany
BSA	Biomol, Hamburg, Germany
Caffeine	Sigma-Aldrich, Deisenhofen, Germany
$CaCl_2$	Sigma-Aldrich, Deisenhofen, Germany
Cell Culture Lysis Reagent 5x	Promega, Mannheim, Germany
DMSO	Roth, Karlsruhe, Germany
EDTA	Roth, Karlsruhe, Germany
EGTA	Sigma-Aldrich, Deisenhofen, Germany
Acetic-acid	Roth, Karlsruhe, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium-bromide	Roth, Karlsruhe, Germany
Glucose	Roth, Karlsruhe, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycin	Roth, Karlsruhe, Germany
Insulin	Serva Feinbiochemica, Heidelberg, Germany
Kanamycin	Merck, Darmstadt, Germany
KCl	Merck, Darmstadt, Germany
Luciferin	Promega, Mannheim, Germany
Methanol	Roth, Karlsruhe, Germany
Na-Acetate	Merck, Darmstadt, Germany
Na-Citrate	Roth, Karlsruhe, Germany
NaCl	Roth, Karlsruhe, Germany
Na_2HPO_4	Serva Feinbiochemica, Heidelberg, Germany
NaOH	Roth, Karlsruhe, Germany
N-Acetyl-L-cysteine	Sigma-Aldrich, Deisenhofen, Germany
Nitrocellulose-Membrane	Amersham Pharmacia, Freiburg, Germany
PBS (1x, sterile)	Invitrogen, Karlsruhe, Germany
Propanol	Roth, Karlsruhe, Germany
Propidium-Iodide	Roth, Karlsruhe, Germany
SDS	Roth, Karlsruhe, Germany

TEMED	Roth, Karlsruhe, Germany
TRIS	Roth, Karlsruhe, Germany
Triton-X-100	Serva Feinbiochemica, Heidelberg, Germany
Trypsin-EDTA (0.05% Trypsin, 0.2 g/l EDTA)	Invitrogen, Karlsruhe, Germany
Trypton	Becton Dickinson, Heidelberg, Germany
Tween	Roth, Karlsruhe, Germany
Water (sterile, distilled)	Pharmacy University Hospital Heidelberg, Germany
Whatman Paper	Whatman, Maidstone, UK

All current laboratory materials not listed here have been purchased from the major suppliers, e.g. Becton Dickinson, Roth, Bio-One, Merck, Greiner.

3. Results

The direct molecular mechanisms through which HBV-infected cells are eliminated by the immune system as well as the signalling pathways up-regulated in infected host cells to combat the virus are still ill-characterized. The present study was designed to investigate both the influence of the hepatocytes' p53 status on the outcome of HBV infection and *vice versa*, the influence of HBV infection on p53 and p53 target gene activation in hepatocytes. The aim was to elucidate factors leading to persistence of HBV and, thus, to HBV-induced hepatocarcinogenesis (viral clearance *vs.* chronicity). The use of different adenoviral HBV vectors (knock-out constructs and the HBV wt construct), two hepatoma cell lines and, of note, primary human hepatocytes allow us to work with an infection model closely related to the *in vivo* situation and to generate clinically relevant data. The following data provide evidence that a functional CD95 apoptosis pathway and an intact p53 status are essential for the elimination of HBV-infected cells by the immune system and that defects in these signalling pathways favours the development of chronic HBV infection and hepatocellular carcinoma. In addition, the role of the intrinsic apoptosis signalling pathway and the important function of the viral HBx protein for viral clearance are described. Finally, the following data indicate that HBV-infected hepatocytes are not only sensitive towards CD95-mediated apoptosis induced by CD95L-secreting lymphocytes, but that HBV-infected hepatocytes are also able to secret CD95L themselves.

3.1 Hepatitis B virus infection, p53 and the extrinsic apoptosis signalling pathway

The tumour suppressor p53 is stabilized and activated amongst others by DDR-induced post-translational phosphorylation in response to cellular stress. In hepatocellular carcinoma (HCC) the p53 pathway is affected at multiple levels. Given that several DNA viruses, including HBV, more precisely the viral HBx protein, trigger or interfere with cellular signalling cascades characteristic of a DDR, the following experiments elucidated whether HBV wt infection leads to the activation of a DDR and therefore stabilization and activation of p53.

3.1.1 Hepatitis B virus infection induces p53 accumulation and activation via the DNA damage pathway

Adenoviral transfer of the different recombinant HBV constructs (AdHBV X-, AdHBV L- and AdHBV wt) into the hepatoma cell lines HepG2 and Hep3B resulted in the accumulation of tumour suppressor p53 in p53$^{+/+}$ HepG2 cells. As expected, no p53 could be detected in the p53$^{-/-}$ Hep3B cells (figure 3.1). Adenoviral transfer of recombinant HBV constructs into primary human hepatocytes (PHHs), as a clinically relevant model, also resulted in the accumulation of p53.
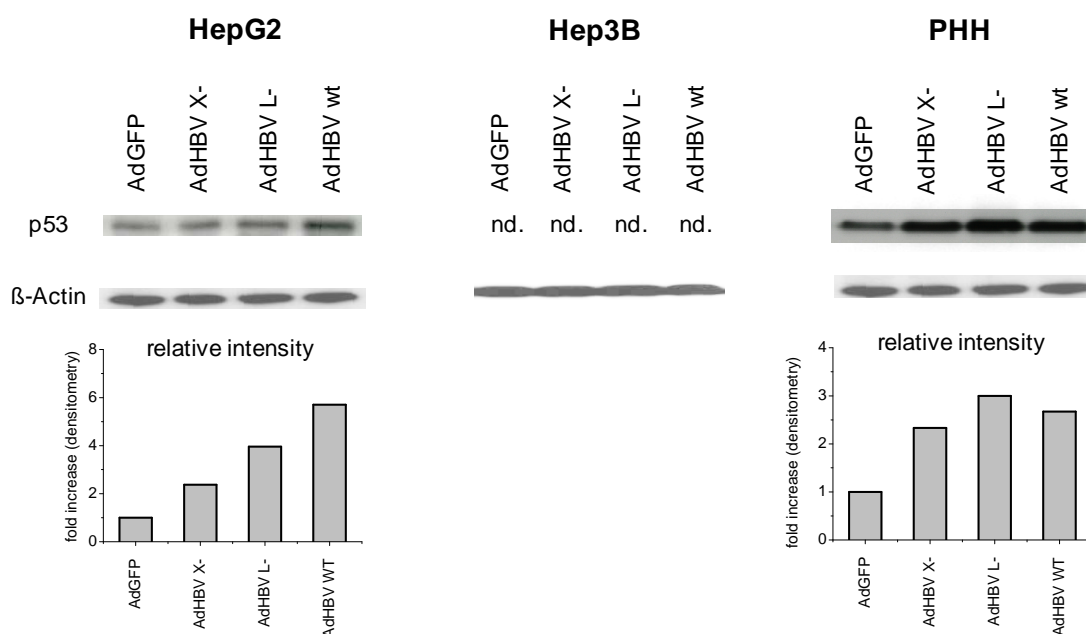
Figure 3.1 HBV infection induces up-regulation of endogenous p53 Western blot analysis of HepG2-, Hep3B-cells 48 h post HBV infection and PHHs 60h post HBV infection, showing an increase in p53 levels in HepG2 cells and PHHs. n.d. = not detected, ß-Actin was used as an internal control.

Based on this observation, the mechanism leading to the stabilization and accumulation of p53 in HBV-infected hepatocytes was investigated. Specifically, p53 was stabilized by Ser^{15} phosphorylation, one of the two serines typically becoming phosphorylated after DNA damage (figure 3.2) (Siliciano, Canman et al. 1997). As several DNA viruses trigger cellular signalling cascades characteristic of a DDR and the HBV protein HBx is known to interfere with the DNA damage pathway, we analyzed whether a cellular DDR is responsible for p53 accumulation observed upon HBV infection. Figure 3.2 shows the phosphorylation status of prominent members of

the DNA damage pathway upon infection with the indicated adenoviral HBV constructs. Adenoviral HBV infection resulted in Ser^{428} phosphorylation of the protein kinase ATR, Thr^{68} phosphorylation of CHK2 and 53BP1 accumulation. The analysis of the phosphorylation status of proteins involved in the DDR revealed cell type specific differences. While, as expected, there was no serine 15 phosphorylation of p53 in Hep3B cells, HepG2 cells and PHHs showed an increased p53 phosphorylation on serine 15. Hep3B cells in contrast, showed the highest increase in threonine 68 phosphorylation of CHK2. In PHHs the phosphorylation of ATR on Ser^{428} was 22-fold higher in cells infected with AdHBV wt than in cells infected AdGFP, an effect which could not been observed in the hepatoma cell lines. The maximal fold increase in hepatoma cell lines was 1.5. The accumulation of 53BP1 was similar in all three cell lines.
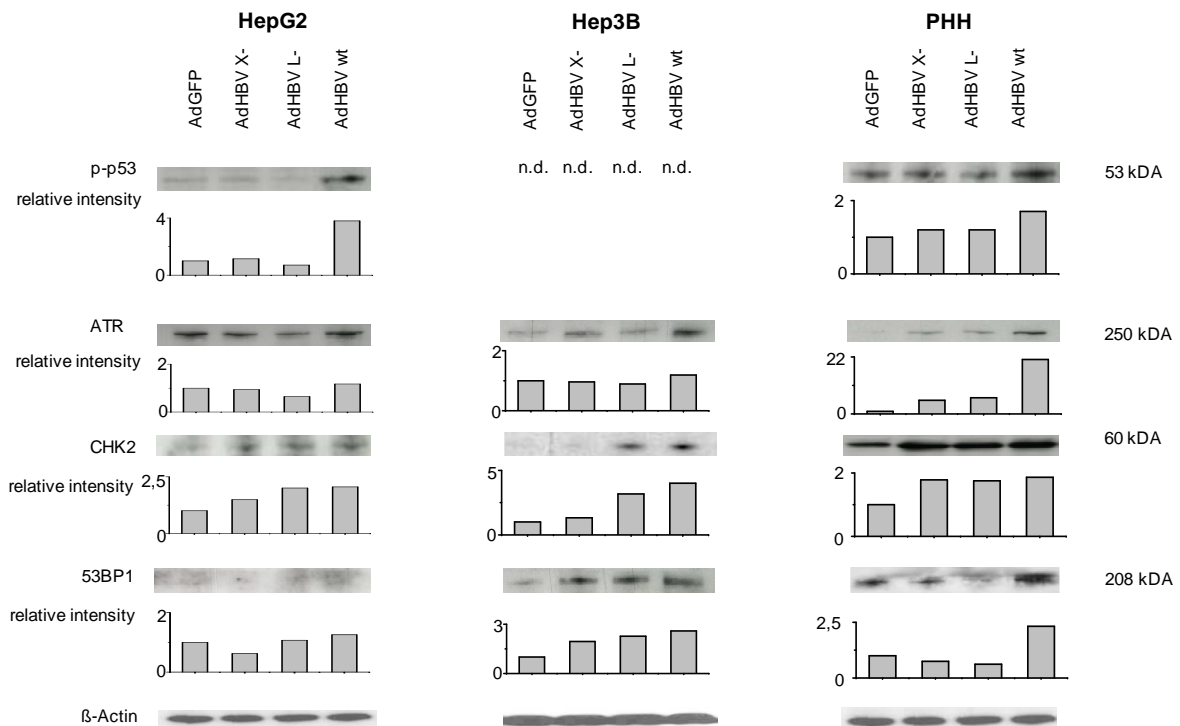
Figure 3.2 HBV infection triggers the activation of the DNA damage response Western blot analysis of HepG2-, Hep3B-cells and PHHs after HBV infection, showing the phosphorylation of proteins involved in the DNA damage response. Protein extracts were prepared as indicated in chapter 2.4.2. For the detection of the phosphorylation status of DNA damage proteins the following time points were used: p-p53 (Ser^{15}), p-ATR (Ser^{428}), p-CHK2 (Thr^{68}) and 53BP1 24 h p.i. (HepG2 and Hep3B); all proteins detected in HBV-infected PHHs were harvested 60 h p.i.; n.d. = not detected, ß-Actin was used as an internal control.

While the HBx-containing adenoviral constructs (AdHBV L- and AdHBV wt) led to an increased phosphorylation of all screened DNA damage proteins, infection with AdHBV X- as well as AdGFP resulted only in a minor or no increase in the phosphorylation status of the proteins.

Furthermore, HBV infection did not only lead to the phosphorylation of p53 on serine 15 but also to Jun-NH$_2$ kinase (JNK) phosphorylation on Thr^{183}/Tyr^{185}. Phosphorylation of JNK is another event leading to the phosphorylation of p53 under cellular stress. The increased phosphorylation of JNK was statistically significant in HepG2 and Hep3B cells. In addition, HBV infection resulted in PARP-1 cleavage, an unalterable marker of apoptosis induction, in the presence and absence of p53 (figure 3.3).
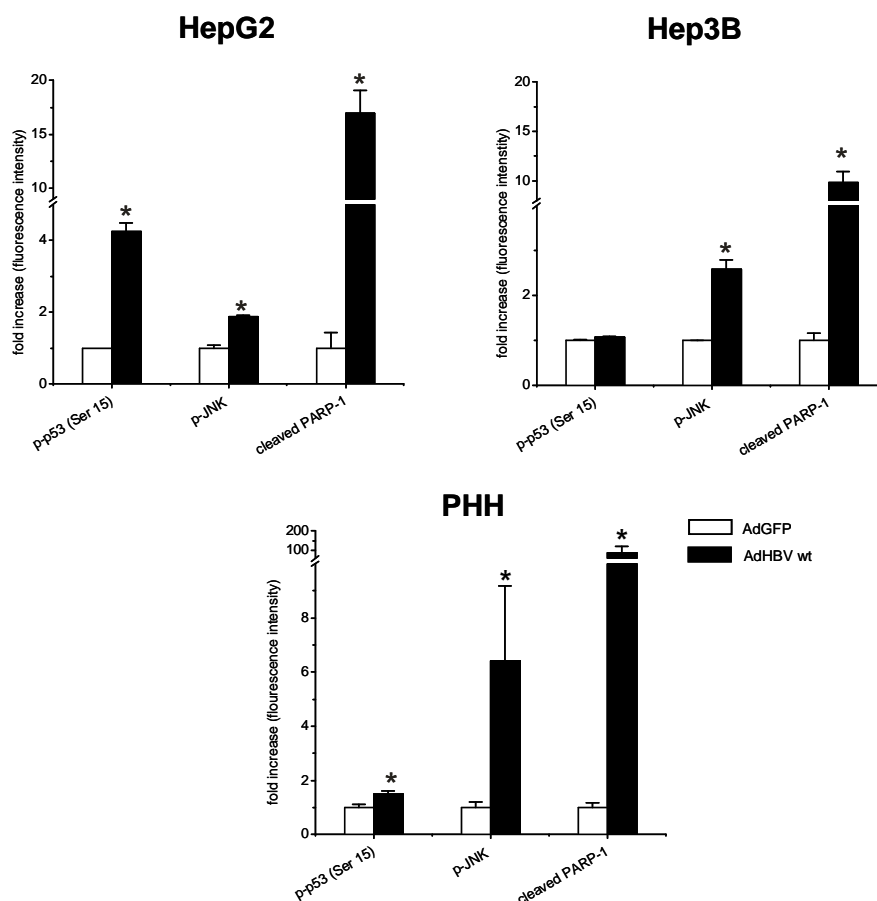
Figure 3.3 Infection of HepG2-, Hep3B-cells and PHHs with AdHBV wt results in the phosphorylation of p53 (p-p53 Ser^{15}), JNK (Thr^{183}/Tyr^{185}) and PARP-1 cleavage Phosphorylation status was determined using Luminex® xMAP® technology (Phospho-Mek, Phospho-Erk and GAPDH were used as internal controls, not shown here). Except for the p-p53 signal in Hep3B cells, the differences between AdGFP and AdHBV wt are statistically significant (*p≤0.05 as determined by Student's t-test).

In summary, these results reveal that HBV infection induces phosphorylation and activation of prominent proteins involved in the DDR, suggesting an integral role of ATM/ATR in the HBV-induced phosphorylation of p53.

3.1.2 Apoptosis induction in HBV-infected hepatocytes is dependent on p53 activation via the DNA damage pathway

In order to investigate whether p53 phosphorylation and accumulation is also associated with its transcriptional activity and stability, the induction of apoptosis in HBV-infected hepatocytes was analysed next (figure 3.4).
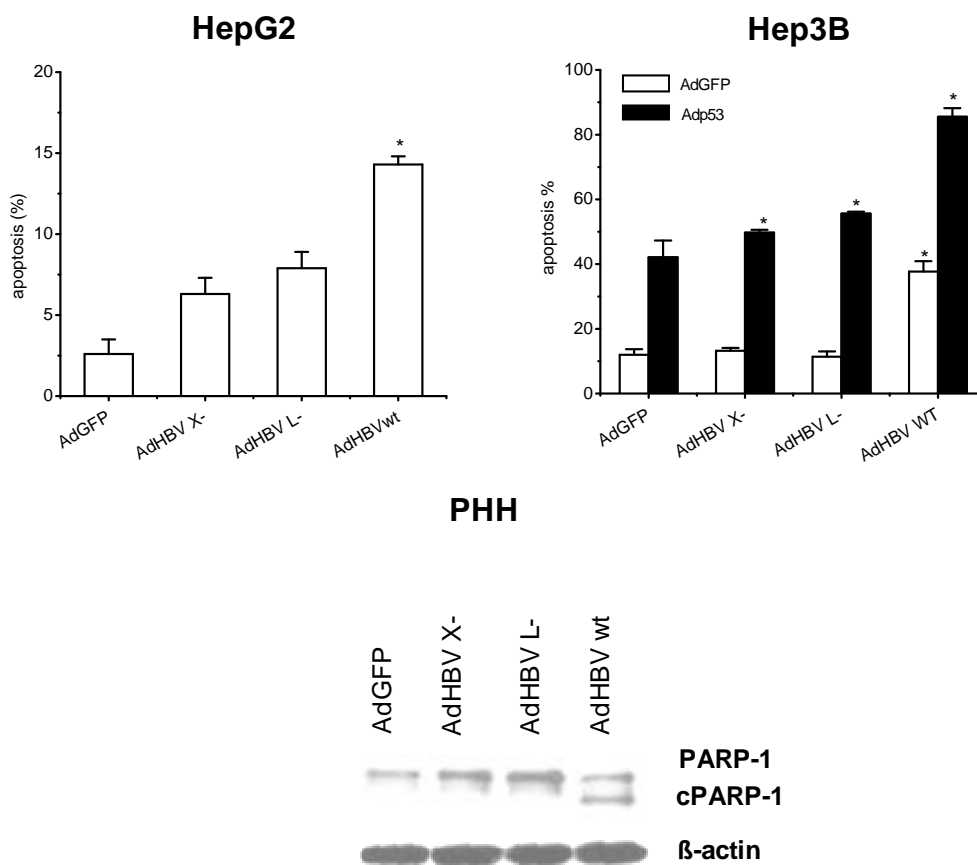
Figure 3.4 Apoptosis of HBV-infected hepatocytes Infection of HepG2-, Hep3B cells and PHHs with AdHBV led to an increased apoptosis induction within 72 h. FACSCalibur[®] analysis of propidium iodide stained nuclei in the subG$_0$/G$_1$ population. Error bars represent standard deviation (Mean ± SD, n=3); one representative experiment out of 3 independent experiments is shown, respectively. The differences between AdGFP and AdHBV wt are statistically significant in HepG2- and Hep3B cells. In Hep3B cells Adp53 addition led to a synergistic effect in apoptosis induction. (*p≤0.05 as determined by Anova, Bonferroni's multiple range test). Western blot analysis of cleaved PARP-1 was used for apoptosis determination at 60 h p.i. in PHHs. ß-Actin served as an internal control.

Infection of HepG2-, Hep3B-cells and PHHs with adenoviral HBV constructs thereby led to an increased apoptosis induction within 72 h analysed by the amount of propidium iodide (PI) stained nuclei in the $subG_0/G_1$ population. The differences between AdGFP and AdHBV wt were statistically significant in HepG2- and Hep3B cells. Combined adenoviral transfer of Adp53 and the HBx-containing adenoviral constructs, AdHBV L- and AdHBV wt, resulted in a synergistic increase of apoptosis in Hep3B cells. In PHHs induction of apoptosis was shown by western blot analysis of cleaved PARP-1. Similar to the results obtained for the hepatoma cell lines the highest amount of apoptotic cells could be observed in PHHs infected with AdHBV wt. Adenoviral transfer of the different HBV constructs led to an accumulation of PARP-1, but in contrast to the other HBV constructs only AdHBV wt triggered the cleavage of PARP-1 and, therefore, apoptosis induction. The induction of apoptosis was markedly less in cells infected with the HBx knock-out construct (AdHBV X-) compared to cells infected with the HBx-containing constructs. Induction of apoptosis by HBV wt was also accompanied by cleavage of PARP-1 in the used hepatoma cell lines (figure 3.3).

To investigate the role of the DDR in the apoptosis induction of HBV-infected hepatocytes, we used the specific ATM/ATR inhibitor caffeine to test whether apoptosis is dependent on the activation of ATM/ATR (figure 3.5). For this, HepG2 and Hep3B cells were treated 24 h prior HBV infection with 5 mM caffeine. The amount of PI positive nuclei in the $subG_0/G_1$ population was analyzed at 96 h p.i. with HBV. The number of propidium iodide stained and, therefore, apoptotic cells was decreased in samples infected with HBV and additionally treated with caffeine. Pre-treatment with caffeine inhibited the induction of apoptosis in HepG2 cells infected with each of the three adenoviral HBV constructs. The amount of apoptotic cells was similar to the amount of apoptotic cells obtained with AdGFP infection. In Hep3B cells, caffeine treatment of cells infected with the AdHBV X- and AdHBV L-constructs also inhibited the induction of apoptosis. In the case of AdHBV wt no effect could be detected, the amount of PI positive cells was almost the same as in cells without caffeine treatment.

Thus, we hypothesise that HBV infection leads to the induction of apoptosis and that apoptosis induction is at least partly dependent on the activation of the DNA damage response, specifically on ATR/ATM.
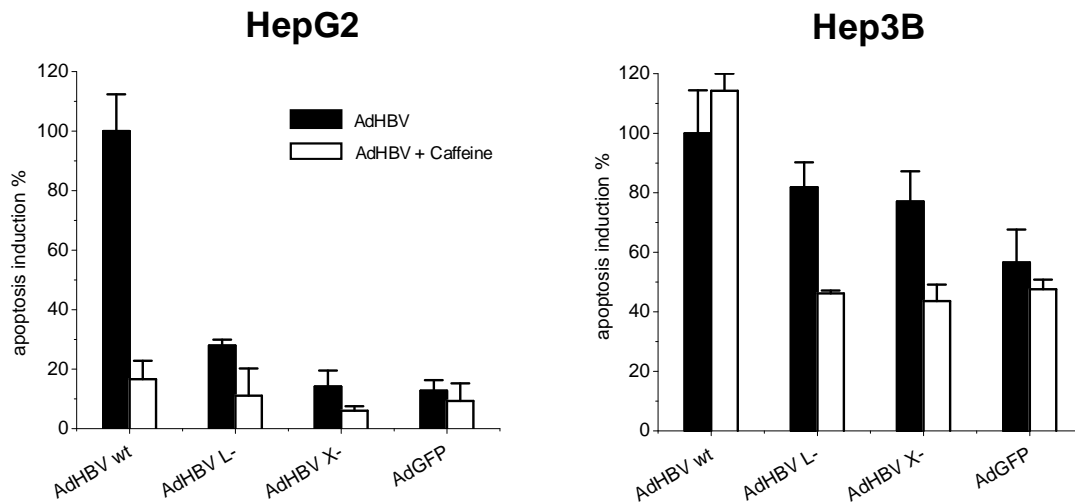
Figure 3.5 Apoptosis induction in HBV-infected hepatocytes is dependent on p53 activation via the DNA damage pathway Treatment of HepG2 and Hep3B cells with caffeine (5 mM), an ATM/ATR inhibitor, prior infection with HBV results in a reduction of HBV-induced apoptosis. The apoptosis rate of hepatocytes infected with AdHBV wt was set to 100%, reduction of apoptosis was calculated accordingly for the other constructs. The amount of propidium iodide positive cells was calculated at 96h p.i. Error bars represent standard deviation (Mean ± SD, n=3).

3.1.3 HBV infection sensitizes hepatocytes towards p53-dependent and CD95-mediated apoptosis

In order to study the role of p53 target genes in the apoptosis of HBV-infected hepatocytes, infected hepatocytes were screened for the activation of prominent p53 target genes involved in apoptosis signalling. One well-described target of p53 playing a pivotal role in the activation of the extrinsic apoptosis signalling pathway is the cell surface death receptor CD95 (Fas). Müller et al. have previously shown that wt p53 transactivates the CD95 gene via binding to a regulatory region within the first intron (figure 3.6a) (Müller, Wilder et al. 1998). In both, Hep3B and HepG2 cell lines, infection with the different AdHBV constructs resulted in an increased CD95 gene activity 24h p.i. as determined by luciferase reporter assay (figure 3.6b). In contrast to HepG2 cells where HBV infection alone was already sufficient to induce CD95 gene transactivation, $p53^{-/-}$ Hep3B cells only show a minor increase in CD95 transactivation. However, in both cell lines infection with AdHBV wt led to a significant increase in gene transactivation. Co-infection of Adp53 with AdHBV in Hep3B cells elevated the CD95 transactivation levels.
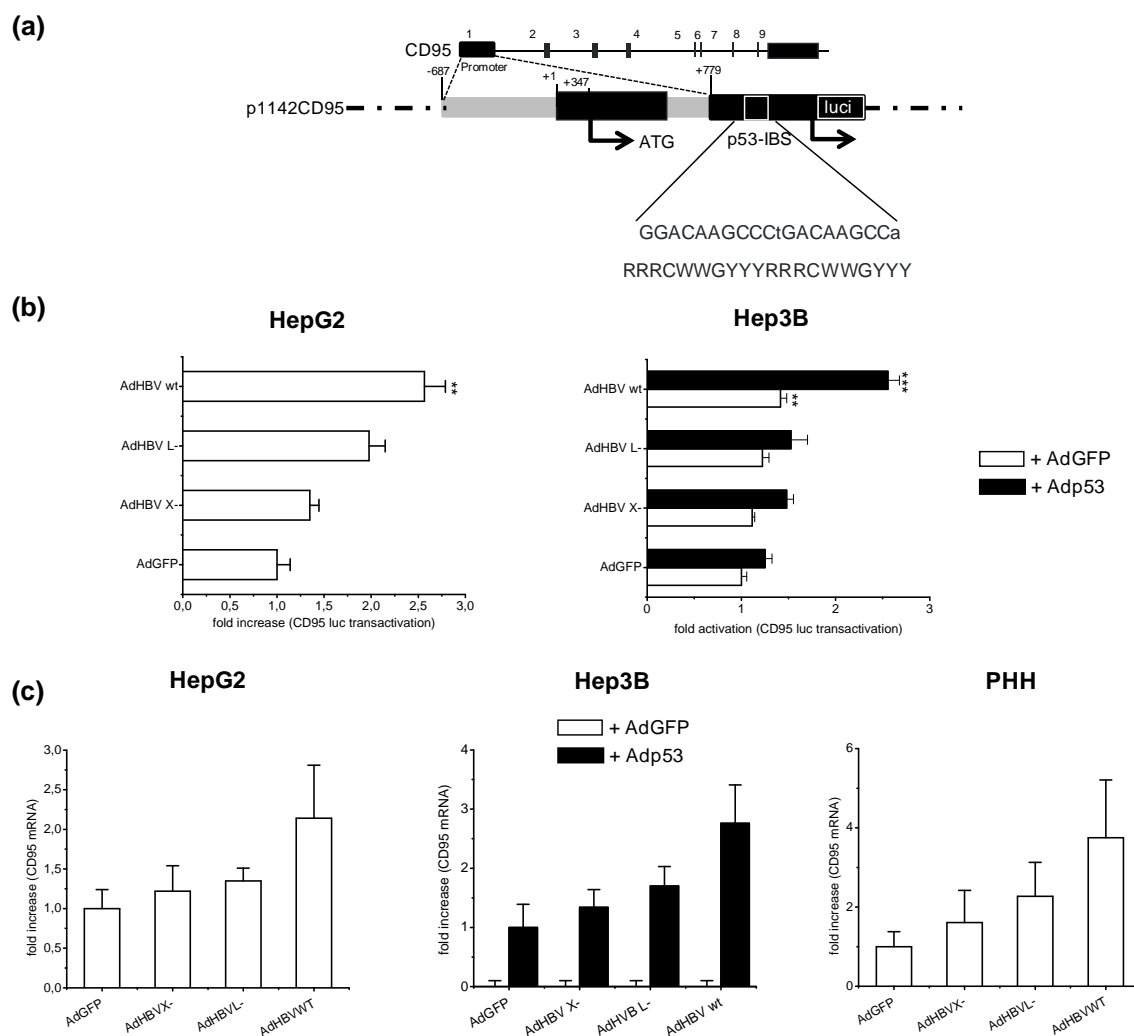
Figure 3.6 HBV infection cooperates with p53 in the transactivation of the CD95 gene, leading to an up-regulation of CD95 mRNA in HBV-infected hepatocytes (a) Map of the human CD95 gene. Exons 1-9 are numbered and represented by black boxes on the upper line. The wild-type sequence (top row) of the intronic p53 binding site (p53-IBS) is shown in comparison to the consensus p53 binding site (bottom row) (R=purine, Y= pyrimidin, W=A or T). **(b)** Analysis of HBV-dependent, CD95 promoter driven luciferase activity. HepG2- and Hep3B cells were transfected with 1 µg wt p1142CD95-luc and infected with 10 MOI of the respective adenoviral HBV constructs. Hep3B-cells were additionally infected with Adp53. Shown is the fold increase of each adenoviral HBV construct, calculated relative to the value obtained with AdGFP. **(c)** Quantification of CD95 mRNA in HBV-infected hepatocytes by real time PCR after 24 h (72 h for PHHs).

One representative experiment out of 3 independent experiments is shown, respectively (Mean ± SD, n=3). The differences between AdGFP and AdHBV wt are statistically significant (**$p \leq 0.01$, ***$p \leq 0.001$ as determined by Anova, Bonferroni's multiple range test).

As a consequence of gene activation, HBV infection led to an up-regulation of CD95 transcription in HepG2 cells (p53$^{+/+}$) and PHH (figure 3.6c). In p53$^{-/-}$ Hep3B cells no CD95 transcription was observed. As CD95 is a typical p53 target gene this was

expected. However, after co-infection of AdHBV and exogenous Adp53, Hep3B cells showed similar CD95 transcription levels as observed in HepG2 cells. Similar to the results described above, CD95 transcription was weaker in hepatocytes infected with HBx negative adenoviral HBV constructs.

Given that HBV infection resulted in the transactivation and transcription of CD95 we were interested to know whether there was also CD95 receptor expression on the surface of HBV-infected hepatocytes. Therefore, receptor expression was determined by FACSCalibur analysis (figure 3.7a). Adenoviral transfer of the different AdHBV constructs and AdGFP as control resulted in an increase in CD95 expression on the surface of HepG2 cells. The difference between AdGFP and AdHBV wt was statistically significant. Addition of exogenous Adp53 led to a synergistic effect in CD95 expression in Hep3B cells infected with the HBx-containing HBV constructs, AdHBV L- and AdHBV wt, which was also statistically significant in the case of AdHBV wt.

Up-regulation of CD95 receptor resulted in an increased responsiveness towards CD95-mediated apoptosis. In fact, treatment with the agonistic, apoptosis-inducing antibody IgG3 anti-APO-1 (AA1) triggered cell death in HBV-infected hepatocytes (figure 3.7b). In HepG2 cells the amount of apoptotic cells increased from basically 20% to almost 70% in the case of AdHBV wt. In contrast, AdHBV X- and AdHBV L-rarely showed a difference in the induction of apoptosis after AA1 treatment compared to AdGFP. However, AdHBV L- clearly induces apoptosis without additional treatment with AA1. In AdHBV- and Adp53-infected Hep3B cells additional treatment with AA1 also resulted in an increase of apoptosis, even if the observed effect was less than in HepG2 cells.
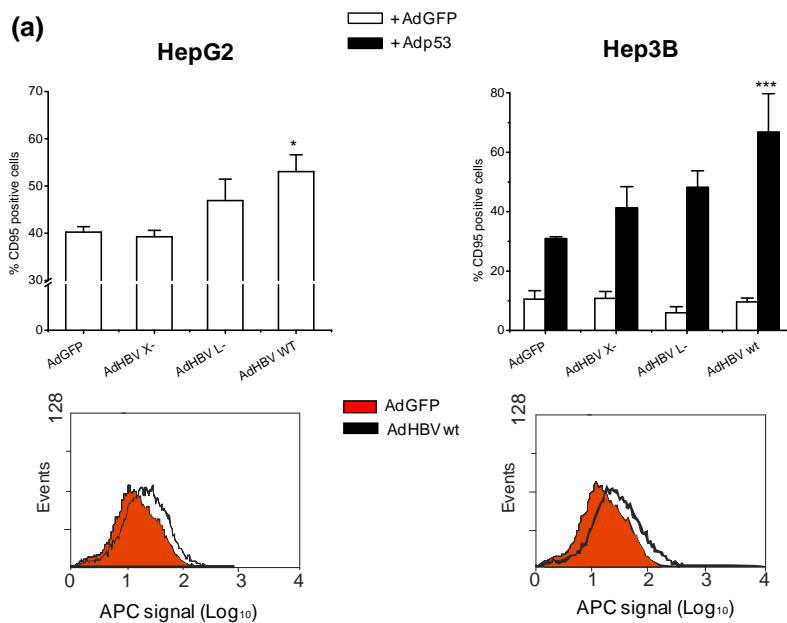
(a)

Figure 3.7 HBV infection together with p53 sensitizes hepatocytes towards CD95-mediated apoptosis (a) Quantitative flow cytometry analysis (FACSCalibur®) of CD95 receptor expression in HBV-infected HepG2- and Hep3B cells. Only HepG2 cells, possessing endogenous p53, display up-regulated CD95 receptor expression. Hep3B cells, lacking p53, were additionally treated with Adp53 to obtain CD95 receptor up-regulation. Percent of CD95 expression was calculated as % CD95$^+$- % isotype control. (*p≤0.05, ***p≤0.001 as determined by Anova, Bonferroni's multiple range test). One representative experiment out of 3 independent experiments is shown (Mean± SD, n=3).

(b) Addition of the CD95 specific agonistic anti-APO-1 antibody (AA1) following HBV infection resulted in the induction of CD95-mediated apoptosis. AA1 was added at 72 h p.i. (post infection) for further 24 h. This led to a significant increase of apoptosis mediated by CD95 in HepG2 cells. In He3B cells Adp53 was co-infected. The figure shows the apoptosis induction of cell treated with Adp53, AdHBV and AA1. Apoptosis was detected by PI staining followed by FACS analysis. The difference in CD95-mediated apoptosis between AdGFP and AdHBV wt infected hepatocytes are statistically significant (*p≤0.05, ***p≤0.001 as determined by Anova, Bonferroni's multiple range test). One representative experiment out of 5 independent experiments is shown (Mean ± SD, n=3).

(b)
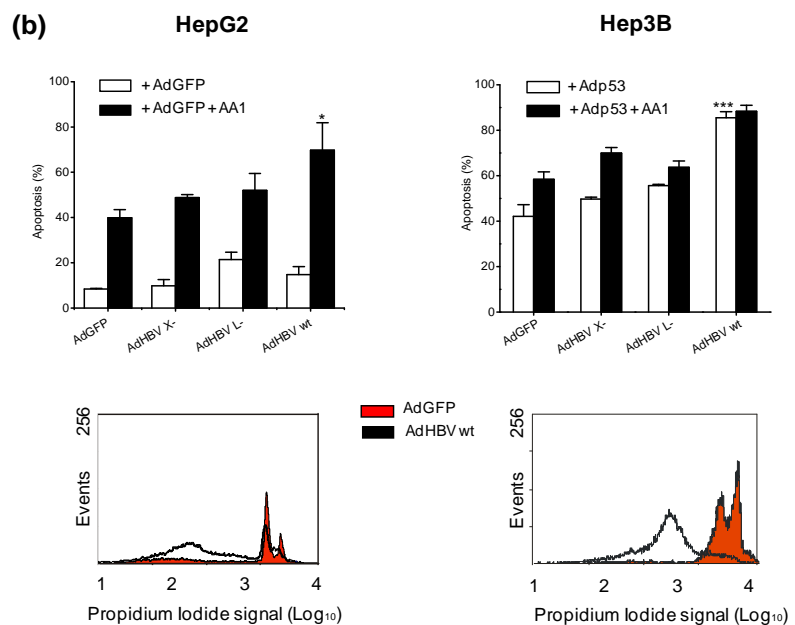
As a result of the experiments obtained with the agonistic antibody IgG3 anti-APO-1 we wanted to look in more detail at the CD95-mediated apoptosis of HBV-infected hepatocytes. Therefore, HBV-infected hepatocytes were treated with the supernatant

of PMA/Ionomycin-treated (P/I) Jurkat 16 cells (SNJ16), which secret soluble CD95L in an increased amount after P/I stimulation, to induce CD95-mediated apoptosis and the induction of apoptosis was monitored. Treatment with SNJ16 resulted in a significant increase in the amount of propidium iodide positive nuclei in the subG$_0$/G$_1$ population (figure 3.8). Up to 60% of the infected hepatocytes underwent CD95-mediated apoptosis after HBV infection and additional treatment with SNJ16. Whereas the apoptosis rate of HepG2 cells did not exceed 40% in total, Hep3B cells benefited from the exogenous Adp53 addition, which led to apoptosis rates between 40 and 60%.
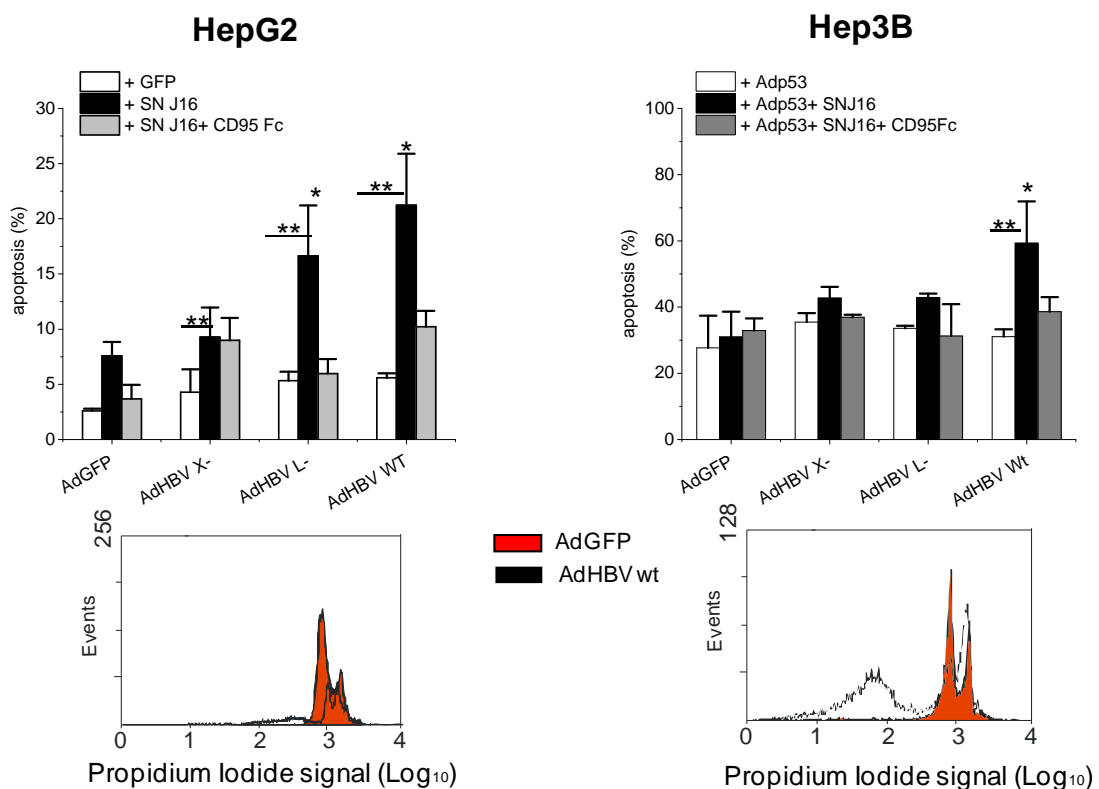
Figure 3.8 HBV-infected hepatocytes are sensitive towards CD95L-mediated apoptosis HepG2-, Hep3B-cells were infected with 10 MOI of the respective adenoviral HBV constructs. In the case of Hep3B-cells Adp53 was added. 72 h p.i. HBV-infected hepatocytes were additionally treated with the supernatant of CD95L-secrecting Jurkat 16 cells (SNJ16), for another 24 h. Apoptosis was detected by propidium iodide stained nuclei. APG101 (10 µg/ml) was used to inhibit CD95-dependent apoptosis. Error bars represent standard deviation of six independent experiments (Mean± SD, n=6). The difference in CD95-mediated apoptosis between AdGFP and AdHBV wt infected hepatocytes is statistically significant (*p≤0.05 compared to AdGFP, **p≤0.01 compared to the next left column, as determined by Anova, Bonferroni's multiple range test).

To verify, that the increased induction of apoptosis was indeed CD95-mediated, we used APG101, a decoy receptor for CD95L (CD95Fc; 10 μg/ml) to specifically inhibit CD95-dependent apoptosis. Additional treatment of HBV-infected and SNJ16-treated hepatocytes with APG101 resulted in a decreased amount of propidium iodide positive cells and, therefore, in inhibition of CD95-mediated apoptosis of HBV-infected hepatocytes.

The effect of CD95L on the HBV-infected hepatocytes was, furthermore, visualized with the help of multicellular tumour spheroids (MCTS). Multicellular tumour spheroids are a well-established 3-D *in vitro* system simulating the pathophysiological *in vivo* situation in tumour microregions. In particular, spheroids can be used as an *in vitro* model to study tumour-immune cell interactions. Due to the results we obtained so far in this work, we show that an intact CD95 apoptosis system is essential for the elimination of HBV-infected hepatocytes. MCTS were used to further analyze and characterize the CD95-mediated apoptosis of HBV-infected hepatocytes by CD95Ligand-secreting lymphocytes.

Adenoviral transfer of the distinct recombinant HBV constructs into MCTS (generated from Hep3B cells) resulted in the inhibition of proliferation as well as the induction of apoptosis of hepatocytes (figure 3.10). To promote CD95-mediated apoptosis, HBV-infected MCTS were treated with the supernatant (SNJ16) of CD95Ligand-secreting lymphocytes (Jurkat 16). While the treatment of MCTS with the supernatant of CD95Ligand-secreting lymphocytes merely led to a reduced hepatocyte-migration out of the spheroid, the treatment of HBV-infected MTCS with SNJ16 resulted in the induction of apoptosis and the disaggregation of the MCTS. This effect was boosted through additional adenoviral transfer of p53 (figure 3.9).

Using MCTS, the interaction between HBV-infected hepatocytes and CD95Ligand secreting lymphocytes could be characterized. CD95Ligand secreting lymphocytes are able to induce CD95-mediated apoptosis in HBV-infected spheroids which confirmed the observations we obtained in experiments with monolayer cells (c.f. figure 3.6 -3.8).
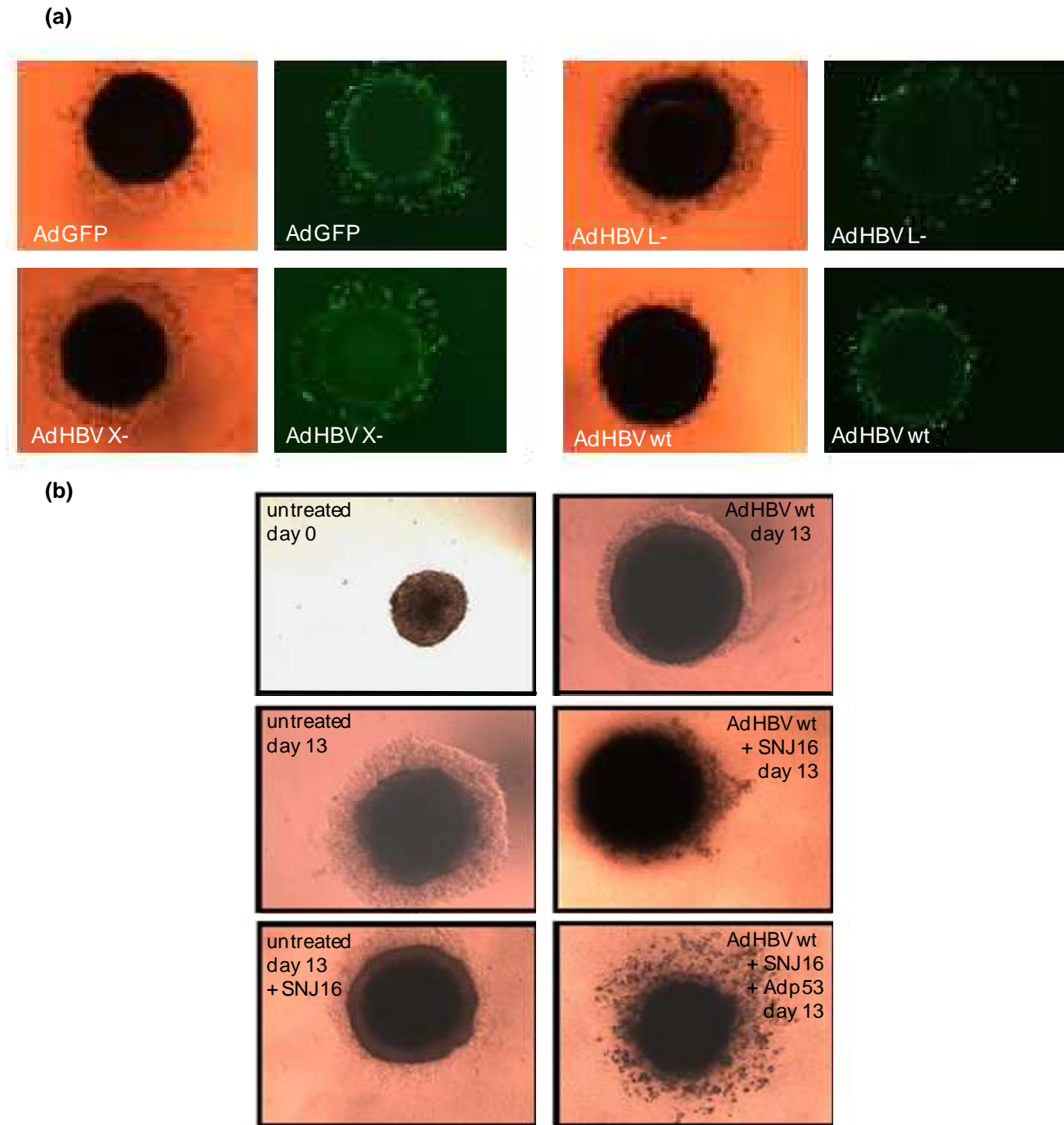
(a)

(b)

Figure 3.9 Multicellular tumour spheroids visualize the pro-apoptotic effect of CD95L-containing supernatant on HBV-infected hepatocytes Spheroids containing 25,000 Hep3B cells were embedded into a collagen matrix and infected with the different recombinant adenoviral HBV constructs and treated or not treated with the supernatant of PMA/Ionomycin stimulated Jurkat 16 cells. **(a)** Spheroids infected with AdGFP, AdHBV X-, AdHBV L- and AdHBV wt in comparison at 24 h p.i. Fluorescence pictures on the right show the amount of infected cells. **(b)** In the picture the different treatments and the subsequent migration reduction can be seen. Cell migration was monitored for 16 days by photographing the spheroids with an inverted Leica phase contrast microscope at 4x magnification at time zero and in 72 h intervals.
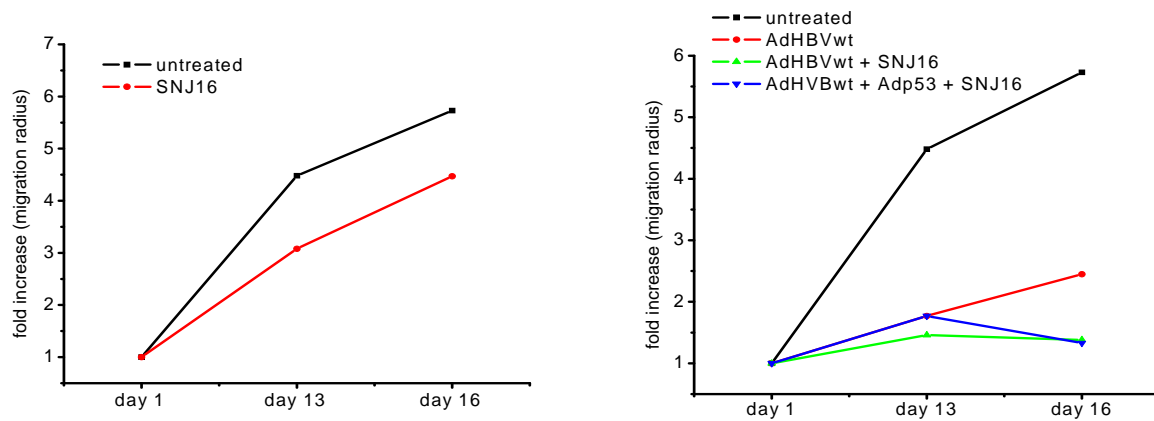
Figure 3.10 HBV infection reduces the migration radius of MCTS Spheroids containing 25,000 Hep3B cells were embedded into a collagen matrix and infected with recombinant adenoviral HBV wt, Adp53 and treated or untreated with the supernatant of PMA/Ionomycin stimulated Jurkat 16 cells. Cell migration was monitored for 16 days by photographing the spheroids with an inverted Leica phase contrast microscope at time zero and in 72h intervals. Calculation of invaded areas was done with Zeiss Axio Vision Release 4.6.3-SP1 software. The size of the untreated spheroid at day 1 was set to 1; the fold increase of cell migration was calculated accordingly.

In summary, these data provide evidence that the apoptosis of HBV-infected cells is mediated by the p53 target gene CD95. Combined adenoviral transfer of Adp53 and AdHBV led to a synergistic transactivation of the CD95 gene, consecutive up-regulation of the CD95 receptor and an increased responsiveness of HBV-infected hepatocytes towards CD95-mediated apoptosis.

3.1.4 HBV infection leads to an increased expression of TRAILR2 in hepatoma cell lines

Beside CD95 various other cell death receptors play an important role in the induction of apoptosis. The p53 target gene TNF related apoptosis inducing ligand receptor 2 (TRAILR2) is one of them. The expression of TRAILR2 was analyzed in HepG2- and Hep3B cells at 72 h post HBV infection (figure 3.11). In contrast to CD95, TRAILR2 was expressed in both hepatoma cell lines. Although, Adp53 was co-infected into Hep3B cells, AdHBV infection alone was sufficient enough to induce TRAILR2 expression. Nevertheless, Adp53 boosted the effect of AdHBV infection. Similar to CD95 expression, the TRAILR2 level was not significantly modulated in cells infected with AdHBV X-.
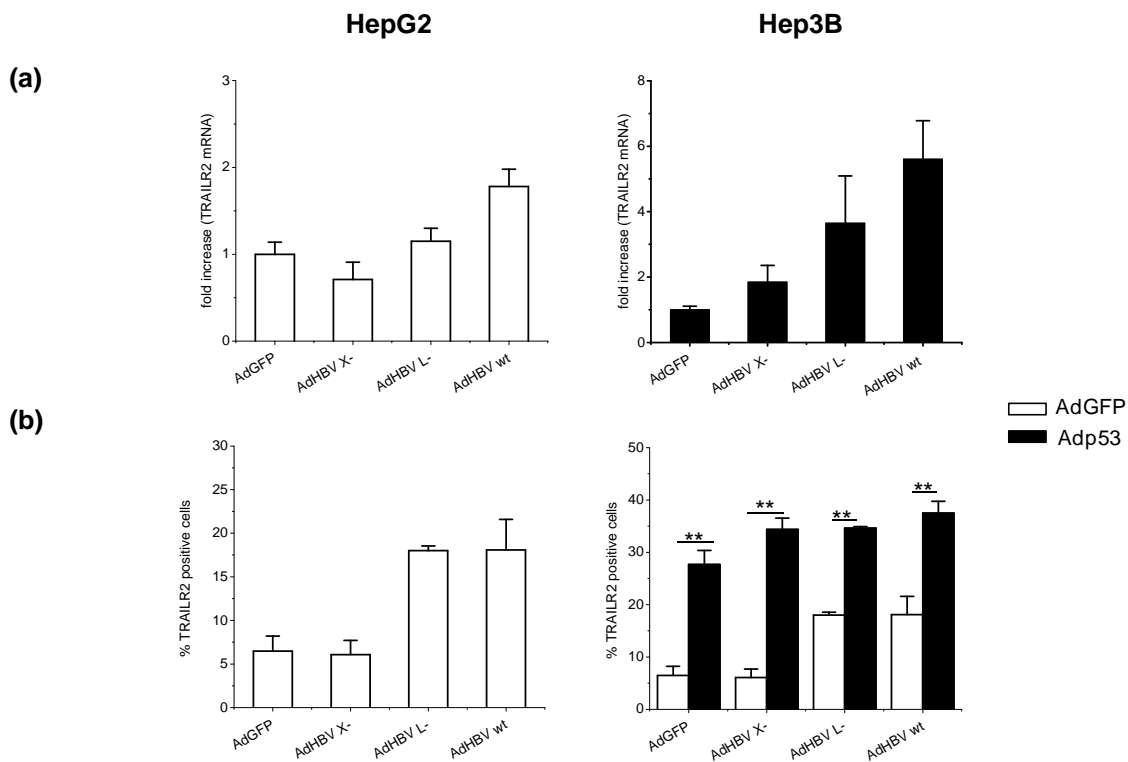

Figure 3.11 In p53$^{-/-}$ Hep3B cells HBV infection results in the up-regulation of TRAILR2 (a) Quantification of TRAILR2 mRNA in HBV-infected hepatocytes by real time PCR after 24 h. **(b)** Quantitative flow cytometry analysis (FACSCalibur®) of TRAILR2 receptor expression in HBV-infected hepatocytes. Percent of TRAILR2 expression was calculated as % TRAILR2^{+}- % isotype control. White bars represent mRNA and accordingly protein expression in HeG2-, and Hep3B-cells infected with AdHBV. Black bars show the effect of Adp53 and AdHBV co-infection on TRAILR2 transcription and expression. Error bars represent standard deviation of 3 independent experiments (Mean± SD, n=3). The difference between AdGFP and Adp53 infected hepatocytes is statistically significant (**p≤0.01 compared to the next left column, as determined by Anova, Bonferroni's multiple range test).

3.1.5 The p53 hot-spot mutant p53R248W inhibits apoptosis of HBV-infected hepatocytes

Whereas the hallmark of wild-type (wt) p53 is its tumour suppressor activity, tumour-associated mutant p53 proteins can exert not only dominant-negative activities but also novel anti-apoptotic "gain-of-function" (GOF) activities, which confer a selective advantage for tumour cells harbouring such mutations (Solomon, Madar et al. 2011). "GOF" mutants of p53 are oncogenic by repressing the activity of genes regulating both the extrinsic and the intrinsic apoptosis signalling pathway. Furthermore, mutant p53 bind and inactivate the TA-isoforms of the p53 family members' p63 and p73, thus, contributing to apoptosis resistance (Schilling, Kairat et al. 2010).

Combined adenoviral transfer of AdHBV and wt p53 into Hep3B cells led to an increased CD95 gene transactivation as well as CD95 protein expression, thus, sensitizing HBV-infected hepatocytes towards CD95-mediated apoptosis (figure 3.12b-d). In contrast, transfection of a p53 hot-spot mutant, namely p53R248W (Ory, Legros et al. 1994), inhibited the viral clearance and apoptosis of HBV-infected hepatocytes. Whereas, wt p53 transactivated the CD95 gene, hence leading to an increased CD95 protein expression on the surface of HBV-infected hepatocytes, p53R248W inhibited CD95 gene transactivation and therefore CD95 protein expression and CD95-dependent apoptosis. In line with the results described so far in this thesis, the HBx-containing constructs show increased levels of CD95 transactivation and expression and therefore a higher amount of apoptotic cells, while AdHBV X- did not differ from the AdGFP level.

These data show that the p53 "gain-of-function" mutant, p53R248W, contributes to viral persistence by inhibiting CD95-mediated apoptosis of HBV-infected hepatocytes. This points to a possible synergy of alterations of the p53 pathway and HBV-induced hepatocarcinogenesis.
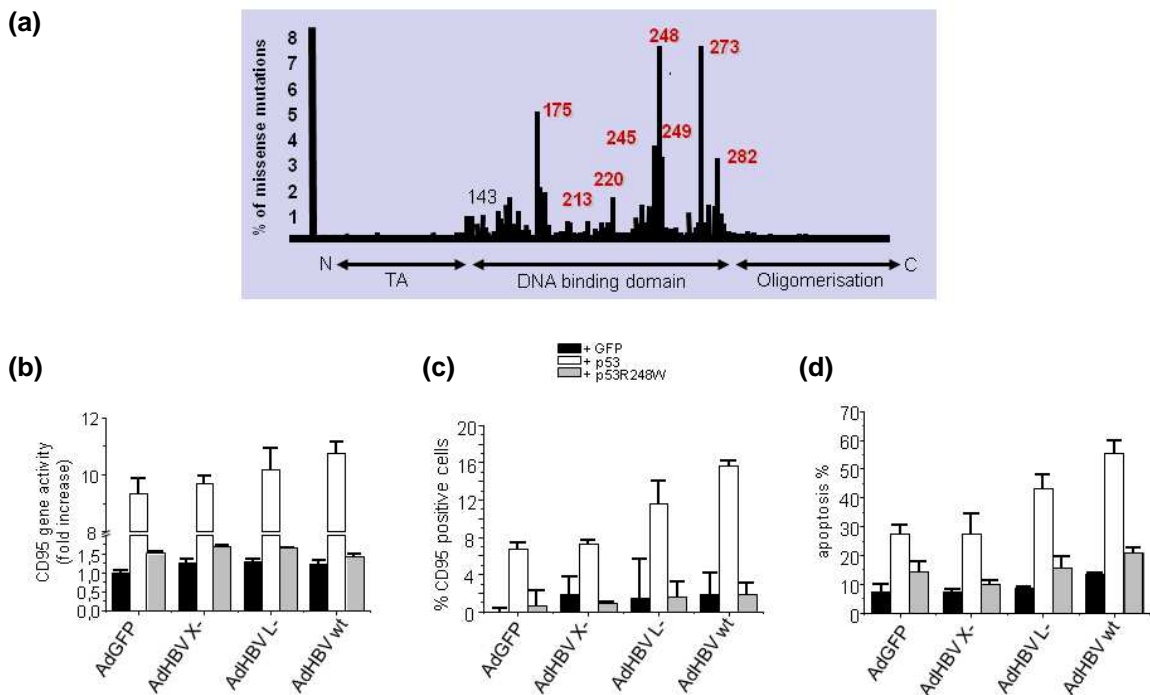
Figure 3.12 p53 hot-spot mutants inhibit apoptosis of HBV-infected hepatocytes

Combined adenoviral transfer of AdHBV and wt p53 into Hep3B cells led to an increased CD95 gene transactivation as well as CD95 protein expression, thus, sensitizing HBV-infected hepatocytes towards CD95-mediated apoptosis. In contrast, transfection of a p53 "GOF" mutant, namely the hot-spot mutant p53R248W, inhibited viral clearance and apoptosis of HBV-infected hepatocytes. **(a)** Distribution of reported missense mutations along the 393 amino-acid sequence of p53. Adapted from Brosh et al. (Brosh and Rotter 2009) **(b)** The hot-spot mutant p53R248W repressed the transcriptional activity of the CD95 gene as detected by luciferase reporter gene assay. Hep3B cells were transiently transfected with 1 µg of p1142CD95-luc together with 100 ng of either wt p53 or p53R248W. **(c)** FACSCalibur analysis of CD95 receptor expression in Hep3B cells following the transient transfection of either wt p53 or p53R248W. **(d)** Wild-type and mutant p53 differentially regulated apoptosis of HBV-infected hepatocytes. FACSCalibur analysis of propidium iodide stained nuclei of Hep3B cells transiently transfected with wt p53 or the hot-spot mutant p53R248W prior infection with HBV.

Values are means ± SD, one representative experiment out of three independent experiments is shown.

3.2 HBV infection triggers AP-1-dependent CD95Ligand expression in HepG2 cells

It has been shown that hepatocytes are not only sensitive towards CD95-mediated apoptosis but also able to express CD95Ligand (CD95L) by themselves and are, therefore, able to eliminate other cells via CD95L-CD95 mediated cytotoxicity (Guy, Wang et al. 2006). Moreover, Eichhorst, Müller et al. demonstrated that chemotherapeutics trigger the transactivation of CD95L in HepG2 cells via an AP-1 element in the promoter of the CD95L gene (Eichhorst, Müller et al. 2000). Having shown that HBV-infected hepatocytes are sensitive towards CD95-mediated apoptosis and taking into account that cytotoxic T cells are, so far, the main effector cells involved in the elimination of HBV-infected hepatocytes, we further investigated the mechanisms of CD95L gene regulation in HBV-infected hepatocytes. In order to study the role of CD95L expression in HBV-infected hepatocytes, different CD95L-luciferase constructs, lacking different regions of the putative binding sites were transfected into HepG2 cells to analyse CD95L transactivation. qPCR analysis and ELISA were performed to study the transcription and expression of CD95L.

3.2.1 Treatment of hepatoma cells with bleomycin leads to the up-regulation of CD95L transactivation

Given that the role of chemotherapeutics in the transactivation of CD95L gene was previously shown (Eichhorst, Müller et al. 2000), bleomycin (30 µg/ml) was used as a positive control for CD95L transactivation. To analyze the effect of bleomycin on the transactivation of the CD95L in hepatoma cells, HepG2 cells were transfected with 1 µg of the following CD95L-luc (-36/+100 CD95L-luc, -220/+100 CD95L-luc, -537/+100 CD95L-luc, -1204/+100 CD95L-luc) constructs, respectively (c.f. Appendix; page 124-125). The four promoter constructs differ in the base pair length 5' of the transcription start site, therefore, containing or lacking specific binding sites (BS), enhancers or inhibitors and possess 100 bp 5' UTR in front of firefly luciferase (figure 3.13).
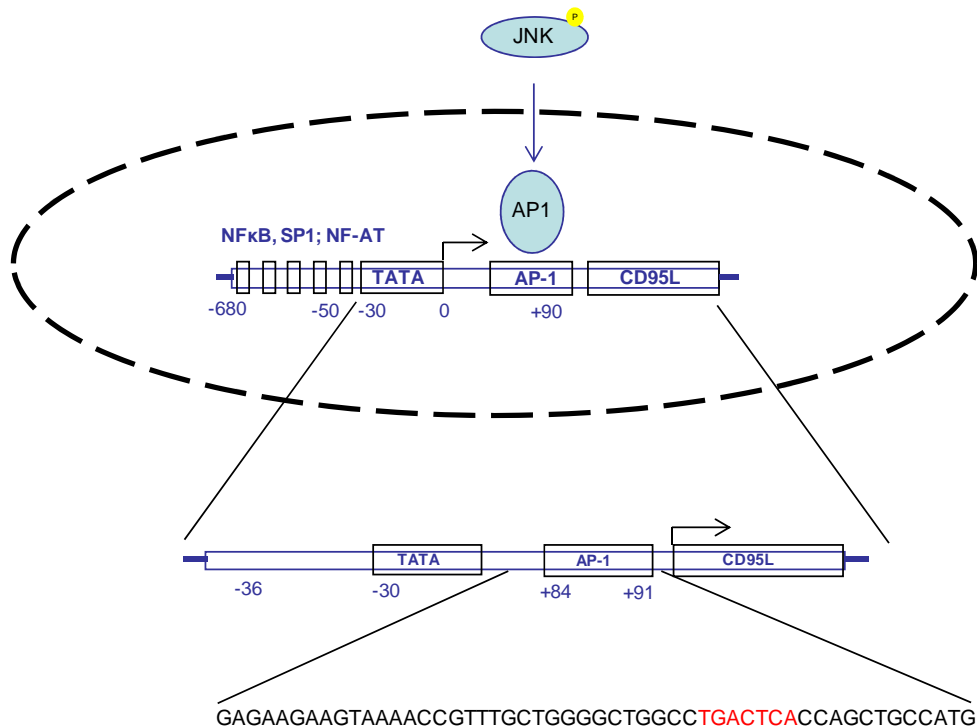
Figure 3.13 Schematic overview of the CD95L promoter Figure was adapted and modified from Li-Weber et al. and Eichhorst et al. (Eichhorst, Müller et al. 2000; Li-Weber and Krammer 2002).

48 h after treatment with 30 µg/ml bleomycin CD95L gene transactivation was assessed by luciferase activity demonstrating a significant difference between treated and untreated hepatocytes. All four CD95L-luc promoter constructs showed an increase in CD95L transactivation after bleomycin treatment (figure 3.14). The highest fold increase in CD95L gene transactivation was observed with the -220/+100 CD95L-luc construct. However, no BS between -36 and -1204 could be identified as the major BS responsible for CD95L transactivation in bleomycin-treated HepG2 cells. To investigate if bleomycin triggers CD95L gene transactivation also via the AP-1 binding site at position + 84 - +91 (5' UTR) as it was already described for other chemotherapeutics from Eichhorst et al. (Eichhorst, Müller et al. 2000), the hepatocytes were pre-treated with 40 nM JNK II inhibitor. The prevention of c-Jun activation and, therefore, the inhibition of AP-1 formation resulted in a decreased transactivation of CD95L gene after bleomycin treatment in all four constructs. To verify the suggestion that the AP-1 BS at position +84 - +91 (5' UTR) in the CD95L gene is indeed responsible for transactivation of the CD95L gene after bleomycin treatment, AP-1 BS deletion constructs were generated and gene transactivation was assayed. The obtained results resembled the results achieved with the JNK II

inhibitor confirming the role of the 5' UTR AP-1 BS in the transactivation of the CD95L gene. Except for the -36/+100 and accordingly the -36/ +81 CD95L-luc construct, the decrease in the fold increase of transactivation following bleomycin treatment was significant after JNK II inhibitor addition or the use of the deletion constructs (figure 3.14).
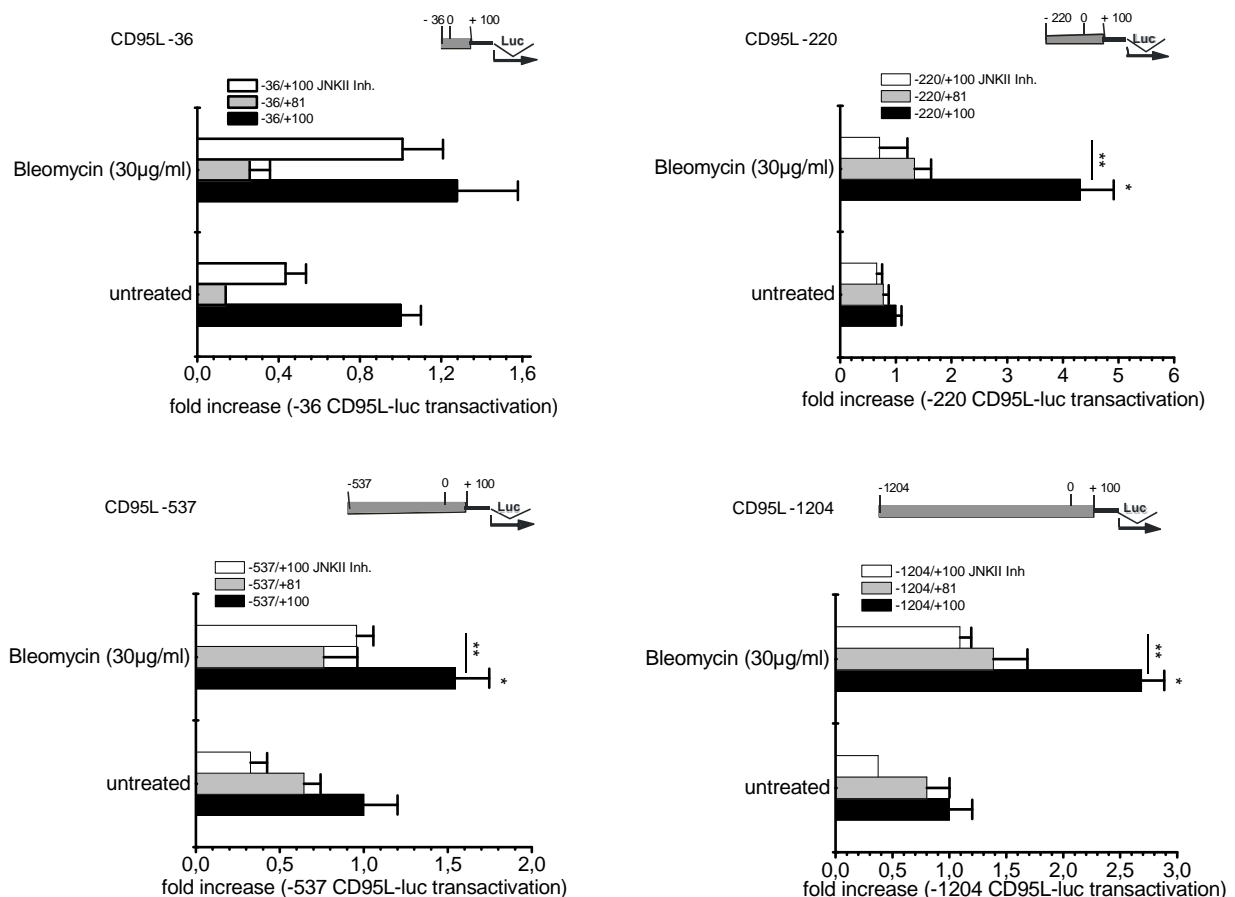

Figure 3.14 Bleomycin treatment results in the transactivation of the CD95L promoter Deletion of the 5' UTR AP-1 BS in the CD95L gene destroys the inducibilty of the four different promoter constructs and induction is inhibited by the JNK II inhibitor. To analyse the effect of the different promoter constructs and the inhibition of JNK on the transactivation of the CD95L promoter after bleomycin treatment (30 µg/ml), HepG2 cells were transfected with the different CD95L luciferase constructs and treated with JNK II inhibitor after transfection with the basal promoter constructs. Following transfection the cells were treated with 30 µg/ml bleomycin for 48 h. Luciferase activity was measured and fold increase was calculated. Luciferase activity measurement in cells transfected with the basal promoter constructs without any treatment was set to 1. One representative experiment out of 3 independent experiments is shown, respectively (Mean ± SD, n=3). (*p≤0.01 compared to untreated samples, **p≤0.01 compared to the AP-1 deletion constructs and JNK II inhibitor treated samples, as determined by Anova, Bonferroni's multiple range test).

To summarize, treatment of the hepatoma cell line HepG2 with the chemotherapeutic drug bleomycin results in the AP-1 dependent transactivation of the CD95L promoter. With the use of the JNK II inhibitor and 5' UTR AP-1 binding site deletion constructs, the AP-1 binding site located between position +84 and + 91 in the 5' UTR of the CD95L gene could be identified as the major transcription factor binding site responsible for the transactivation of CD95L gene upon bleomycin treatment.

3.2.2 HBV infection results in the transactivation, transcription and expression of CD95L in HepG2 cells

Having established that bleomycin leads to the transactivation of CD95L in HepG2 cells, we analyzed whether HBV infection has a similar effect on the transactivation of the CD95L gene. Thus, CD95L transactivation, transcription and expression in HBV-infected HepG2 cells were assessed. First of all, the amount of CD95L mRNA in HBV-infected HepG2 cells was measured and compared to samples treated with bleomycin. Cells were treated with the respective adenoviral HBV constructs or treated with 30 µg/ml bleomycin. Cells were lysed at 36 h p.i. and accordingly post treatment and the amount of CD95L mRNA was analyzed. Both, adenoviral transfer of recombinant AdHBV constructs and treatment with bleomycin led to an increased transcription of the CD95L gene compared to infection with AdGFP and accordingly untreated samples (figure 3.15a). Since there was almost no difference in the induction of CD95L between the untreated cells and the cells infected with AdGFP, CD95L gene transactivation due to adenoviral infection itself was obviated. In accordance with the results described so far in the present study, the highest transcription of the CD95L gene was obtained with the HBx-containing constructs, AdHBV L- and AdHBV wt. Both construct also outranged the fold increase achieved with bleomycin treatment.

In order to study the expression of CD95L two different methods were used. The most sensitive method is the detection of CD95L with an ELISA assay. Therefore, cells were infected with the distinct adenoviral constructs or treated with bleomycin. To analyze the amount of soluble (sCD95L) and membrane-bound CD95L (mCD95L) supernatants were collected and cell lysates were prepared at 72 h p.i. (figure 3.15b). The supernatant of PMA/Ionomycin stimulated Jurkat 16 cells served as positive control for sCD95L expression.
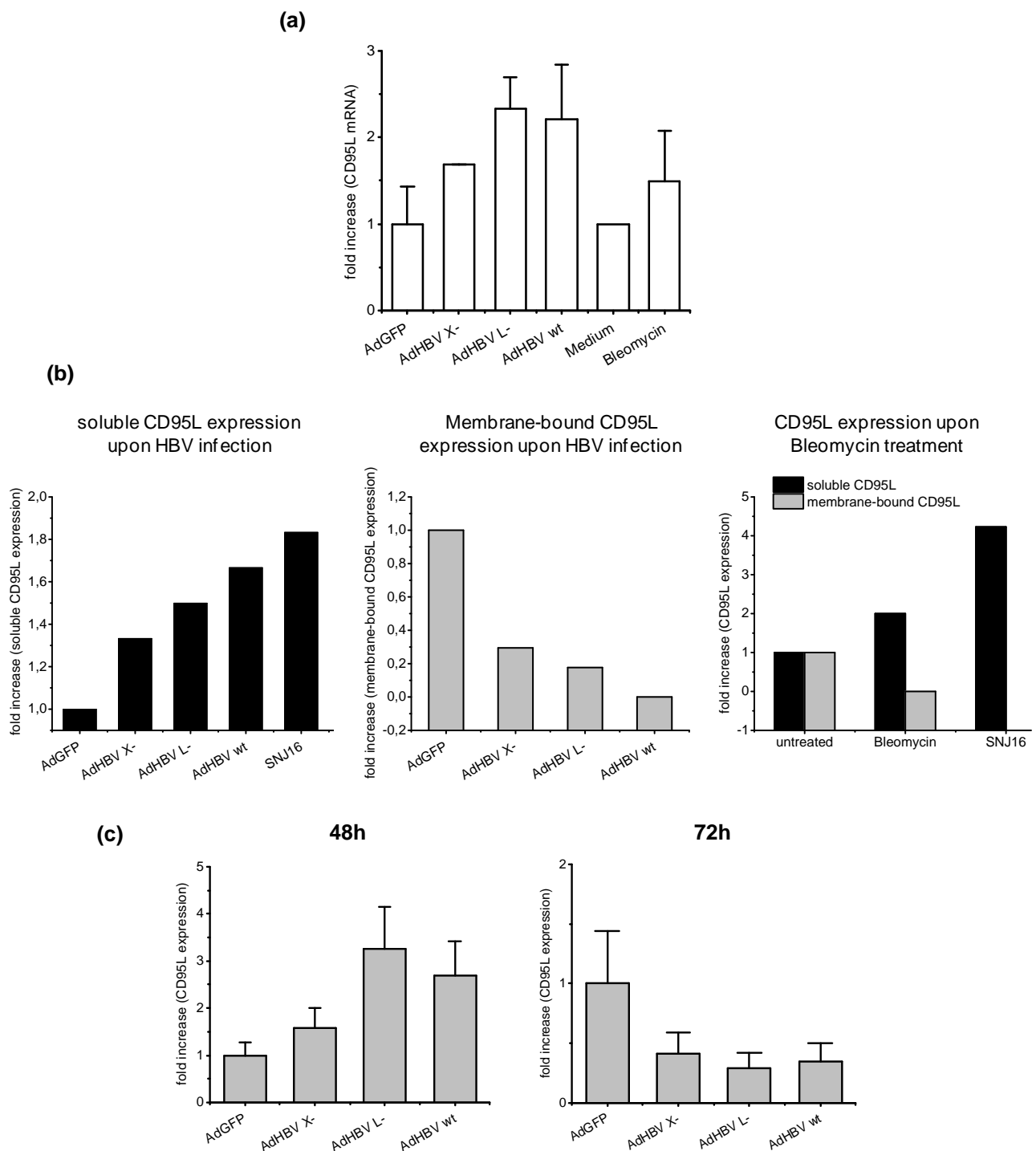
(a)

(b)

(c)

Figure 3.15 HBV results in the transcription of the CD95L gene which leads to the up-regulation of CD95L on the cell surface of HepG2 cells (a) Quantification of CD95L expression after HBV infection by real time PCR in HepG2-cells (24 h p.i.). Total RNA was extracted and qPCR was performed as described in Materials and Methods. One representative experiment out of at least 3 independent experiments is shown, respectively. **(b)** CD95L ELISA showing the fold increase of soluble and membrane-bound CD95L 72 h p.i.; shown is one representative experiment out of three independent experiments. **(c)** Quantitative flow cytometry analysis (FACSCalibur®) of CD95L expression in HBV-infected HepG2 cells 48 h and 72 h p.i. Percent of CD95L expression was calculated as % CD95L^{+}- % isotype control. The fold increase of AdGFP was set to 1. Shown is mean ± SD, n= 3.

Adenoviral transfer of recombinant HBV vectors led to increased levels of soluble CD95L (sCD95L) in HepG2 cells. In line with AdHBV infection, bleomycin treatment also resulted in augmentation of sCD95L. While the amount of soluble CD95L increased upon HBV infection or bleomycin treatment compared to infection with AdGFP or untreated samples respectively, the amount of mCD95L decreased. In the case of AdHBV wt and bleomycin no detectable amounts of mCD95L could be measured. The decrease in the amount of mCD95L was oppositional to the increase of sCD95L upon infection or respectively bleomycin treatment. Another method to analyze the amount of CD95L protein expression is FACS analysis. Thus, infected cells were stained with the corresponding antibody at 48 h and 72 h p.i. and analyzed by FACSCalibur. In contrast to the CD95L ELISA, FACS analysis only detects the amount of membrane-bound CD95L. FACS analysis showed an increase in mCD95L expression upon infection with AdHBV L- and AdHBV wt at 48 h p.i. After another 24 h (72 h post infection) no mCD95L expression could be detected any more. These results, in combination with the results of the mCD95L ELISA, suggest that mCD95L expressed on the surface of hepatocytes is cleaved after 48 h. Figure 3.15c shows the elevated levels of mCD95L expression at 48 h upon HBV infection, as well as the decrease in mCD95L expression at 72 h post infection.

To further analyse the upstream signalling cascade, leading to the transcription and expression of CD95L after HBV infection, CD95L-luc assays were performed.
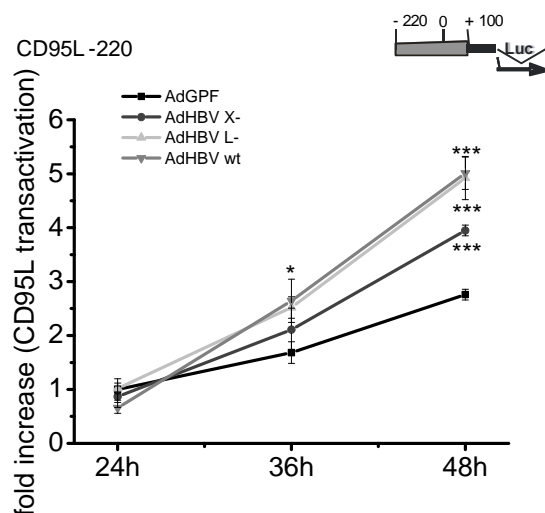

Figure 3.16 Time-course of CD95L gene transactivation after HBV infection HepG2 cells were transiently transfected with 1 µg of the -220/+100CD95L-luc promoter construct. Cells were additionally infected with 10 MOI of the different AdHBV constructs, respectively. Cells were harvested and analyzed after 24 h, 36 h and 48 h to analyze time-dependent CD95L transactivation after HBV infection. (Mean ± SD, n=6). *p≤0.05, ***p≤0.001 compared to AdGFP as determined by Anova.

Time kinetics of CD95L transactivation analyzing the transactivation of the -220/+100 CD95L-luc reporter gene at 24 h, 48 h and 72 h post HBV infection were used to indicate the best time-point for analysis.

In contrast to cells infected with HBV for 24 h, where there was no increase in CD95L gene transactivation detectable, lysates from cells infected for 36 h or 48 h showed an augmented fold increase. While the significance standard between the diverse HBV constructs 36 h p.i. was only $p \leq 0.05$ the fold increase of the diverse constructs 48 h p.i. showed significance standards $p \leq 0.001$ compared to AdGFP (figure 3.16). Thus, the 48 h infection period was considered as an optimal time-point for the following transactivation studies.
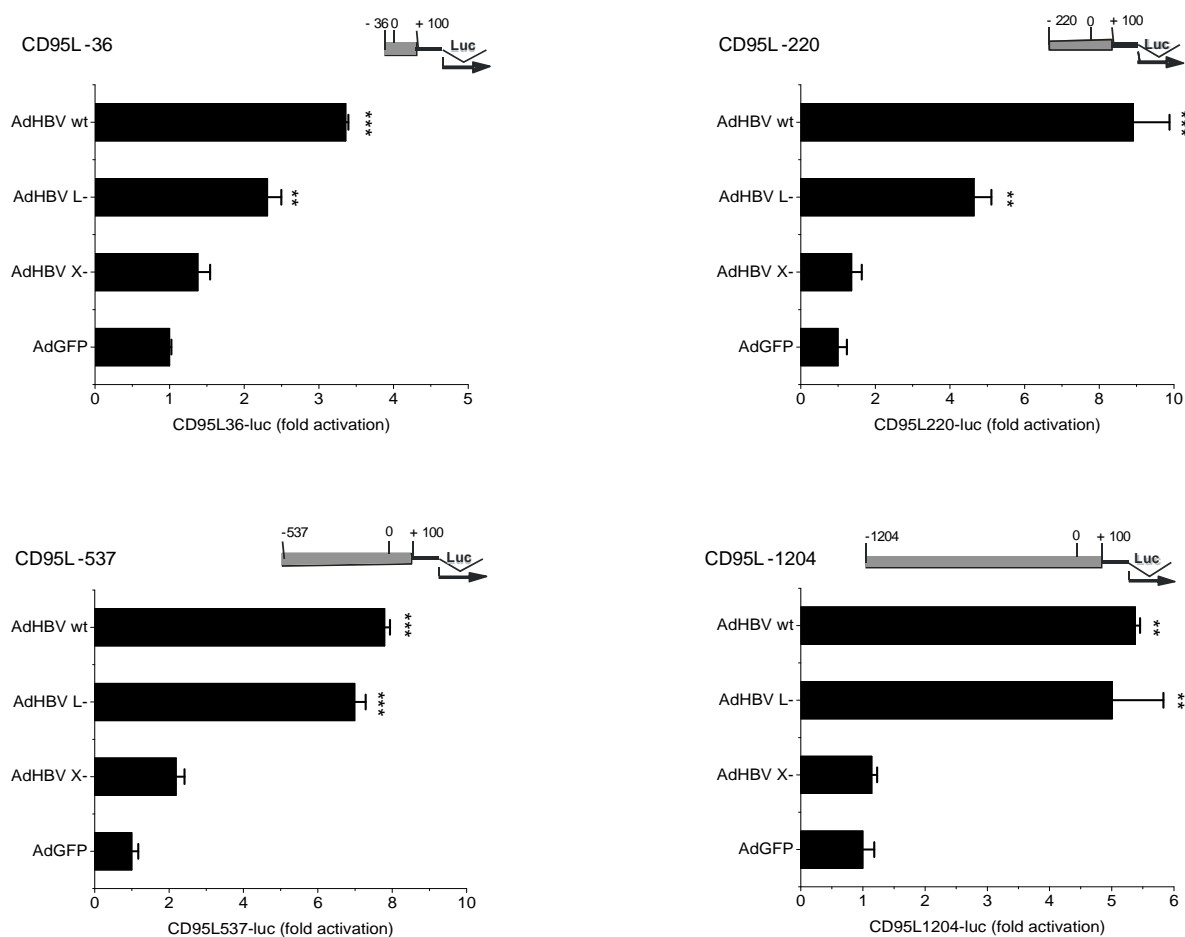

Figure 3.17 HBV infection leads to the transactivation of the CD95L gene HepG2 cells were transiently transfected with 1 µg of the following CD95L promoter constructs: -36/+100CD95L-luc, -220/+100CD95L-luc, -537/+100CD95L-luc and -1204/+100CD95L-luc. Cells were additionally infected with 10 MOI of the different AdHBV constructs, respectively. One representative experiment out of at least 3 independent experiments is shown, respectively. The differences between AdGFP and AdHBV are statistically significant (Mean ± SD, n=3). Luciferase activity value obtained with AdGFP was set to 1. (**p≤0.01, ***p≤0.001 as determined by Anova, Bonferroni's multiple range test).

The distinct CD95L-luc promoter constructs (CD95L -36/+100, -220/+100, -536/+100, and -1204/+100) have been used to identify the binding site responsible for CD95L transactivation in HBV-infected hepatocytes (figure 3.17). AdHBV L- and AdHBV wt infection resulted in a significantly increased fold increase in all four different promoter constructs compared to infection with AdGFP.
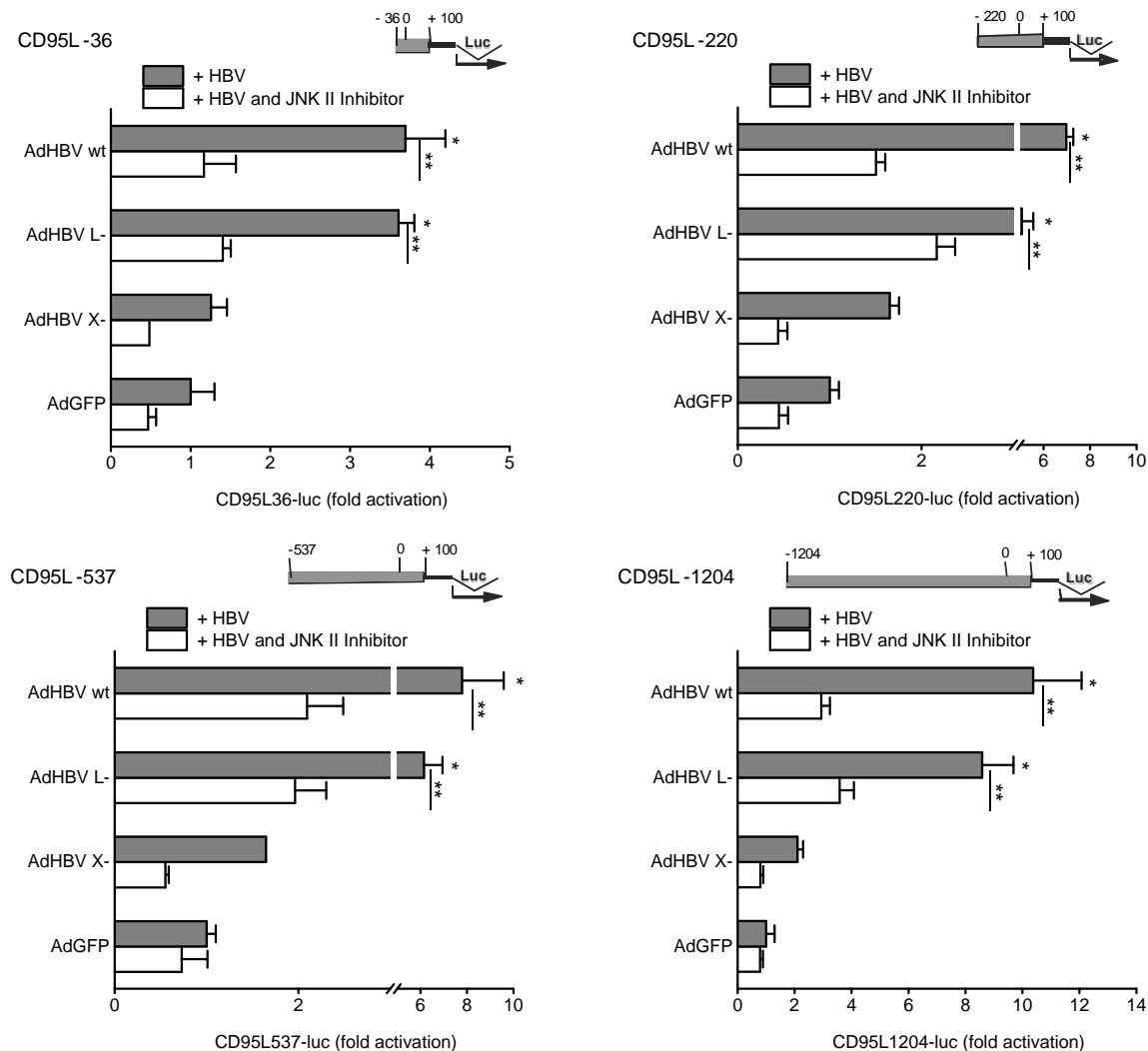

Figure 3.18 The transcription factor AP-1 is responsible for CD95L transactivation in HBV-infected cells HepG2 cells were transiently transfected with 1 µg of the following CD95L promoter constructs: -36/+100CD95L-luc, -220/+100CD95L-luc, -537/+100CD95L-luc and -1204/+100CD95L-luc as indicated. Cells were additionally infected with 10 MOI of the different AdHBV constructs, respectively. 40 nM JNK II Inhibitor was added as indicated. One representative experiment out of at least 3 independent experiments is shown, respectively. The differences between AdGFP and AdHBV are statistically significant (Mean ± SD, n=3). Luciferase activity value obtained with AdGFP was set to 1. (*p≤0.01 compared to untreated, **p≤0.01 compared to JNK inhibitor, as determined by Anova, Bonferroni's multiple range test).

In general, the fold increase was higher compared to the bleomycin-treated samples. However, there was no particular difference in the fold increase of the distinct CD95L-luc-reporter constructs. While the HBx-containing HBV constructs led to an increased CD95L gene transactivation, the HBx knock-out construct, AdHBV X-, did only marginally differ from AdGFP. The HBx-containing HBV constructs also led to an increase of endogenous CD95L transcription and translation (c.f. figure 3.15).

Even though the CD95L-luc transactivation differed from 4-fold to up to 9-fold with AdHBV wt, no binding site between -36 and -1204 could be assigned as the major binding site responsible for CD95L transactivation in HBV-infected hepatocytes. Instead, inhibition of JNK (Jun NH_2-terminal kinase) resulted in a significant decrease in CD95L transactivation (figure 3.18). Inhibition of JNK with a JNK II inhibitor was used to investigate the role of JNK, and in turn the function of the AP-1 BS at position +84 - +91 in the 5' UTR of the CD95L gene. Pre-treatment of HepG2 cells with the JNK II inhibitor resulted in the reduction of CD95L gene transactivation in HBV-infected cells. That CD95L transactivation was only significantly reduced in cells infected with AdHBV L- and AdHBV wt can be explained with the non-significant gene transactivation in AdHBV X- infected hepatoma cells.

Taking the fact into account that the use of a JNK II inhibitor results in the inhibition of CD95L transactivation and that HBV infection led to the phosphorylation of JNK and accordingly to the subsequent activation of the transcription factor AP-1, we postulated that CD95L transactivation is triggered by Thr^{183}/Tyr^{185} phosphorylation of JNK and the subsequent activation of AP-1. The phosphorylation status of JNK upon HBV infection in HepG2 cells is shown in figure 3.19 as well as in figure 3.3.
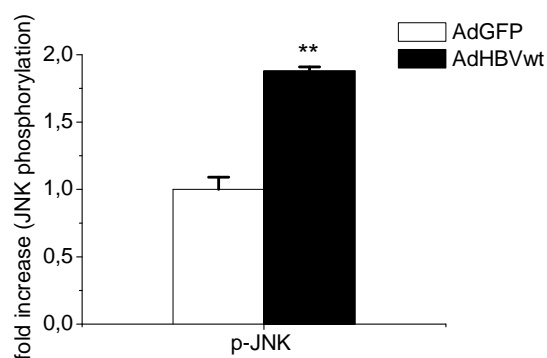

Figure 3.19 HBV infection leads to the phosphorylation of the stress-activated Jun NH2-terminal kinase HepG2 cells were infected with 10 MOI of the different AdHBV constructs, respectively. (Phospho-Mek, Phospho-Erk and GAPDH are internal controls, not shown here). One representative experiment out of at least 3 independent experiments is shown, respectively. The difference between AdGFP and AdHBV is statistically significant (**$p \leq 0.01$ as determined by the Student's t-test).

To confirm the predominant role of the AP-1 binding site in the transactivation of the CD95L promoter we established 5' UTR AP-1 binding site deletion constructs.

While HBV-infected hepatocytes transfected with wt CD95L-luc reporter constructs showed an increased CD95L-luc gene transactivation hepatocytes transfected with CD95L-luc constructs lacking the 5' UTR AP-1 binding site, namely -36/+81CD95L-luc, -220/+81CD95L-luc, -536/+81CD95L-luc and -1204/+81CD95L-luc, displayed a lack in the induction of CD95L transactivation upon HBV infection.
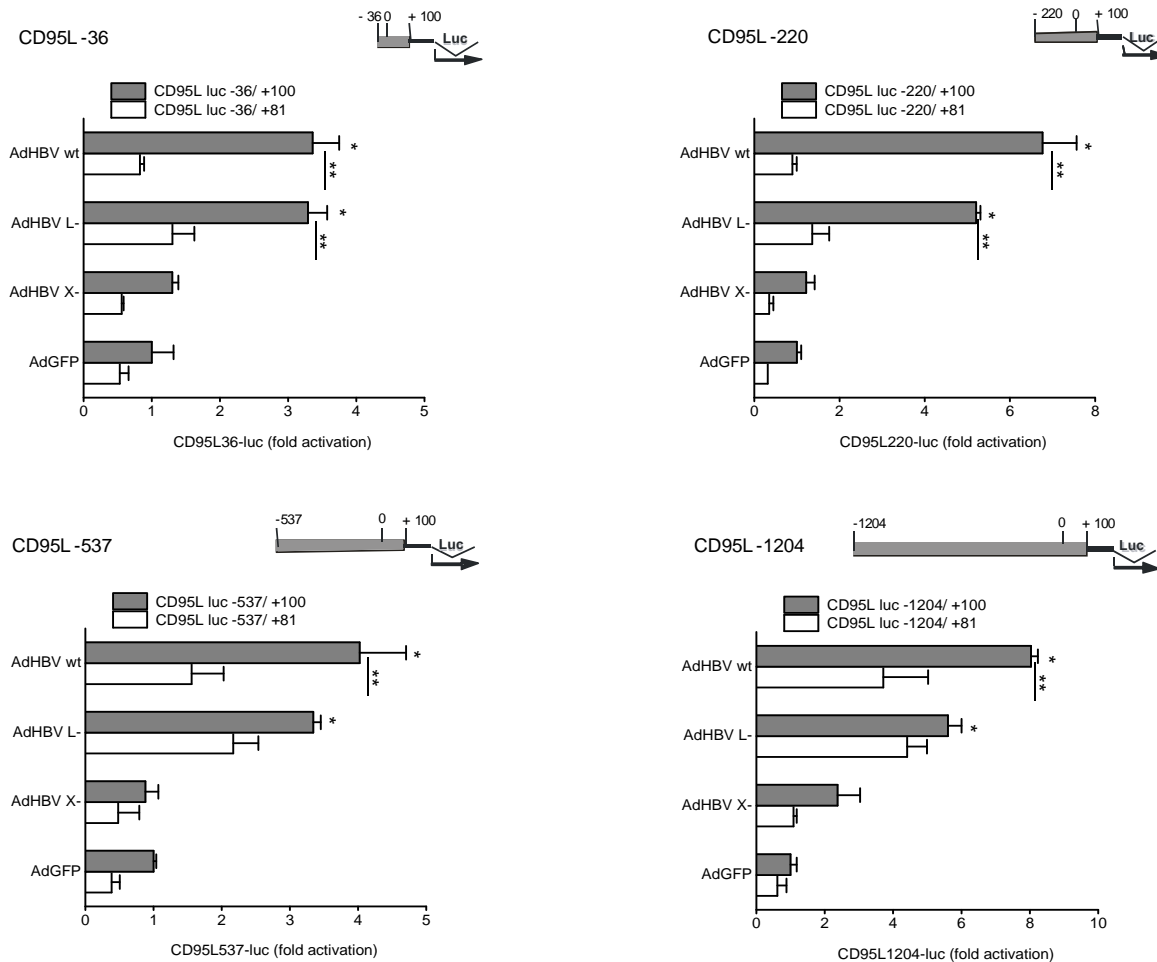

Figure 3.20 CD95L transactivation upon HBV infection is dependent on the AP-1 binding site in the promoter region HBV infection leads to the transactivation of the CD95L gene in HepG2 cells. Cells were transiently transfected with 1 µg of the following CD95L promoter constructs: -36/+100CD95L-luc, -220/+100CD95L-luc, -537/+100CD95L-luc and -1204/+100CD95L-luc or -36/+81CD95L-luc, -220/+81CD95L-luc, -537/+81CD95L-luc and -1204/+81CD95L-luc as indicated. Cells were additionally infected with 10 MOI of the different AdHBV constructs, respectively. One representative experiment out of 3 independent experiments is shown, respectively. The differences between AdGFP and AdHBV are statistically significant (Mean ± SD, n=3). Luciferase activity value obtained with AdGFP was set to 1. (*p≤0.01 compared to untreated, **p≤0.01 compared to the next right column, as determined by Anova, Bonferroni's multiple range test).

Whereas the CD95L-luc promoter gene was transactivated in the promoter constructs containing the 5' UTR AP-1 BS, which suggests an increased CD95L protein expression driven from the endogenous promoter on the surface of HBV-infected hepatocytes, 5' UTR AP-1 binding site deletion constructs failed to induce a sufficient CD95L-luc promoter gene transactivation (figure 3.20). This suggests that the AP-1 BS at position 5' UTR +84 - +91 is necessary for the expression of the endogenous CD95L gene upon infection with HBV.

To investigate the upstream mechanisms leading to the activation of JNK and/or the later transactivation of CD95L gene, different cytokines and chemically reactive molecules were screened for their ability to induce CD95L. The tumour necrosis factor alpha (TNF-α) and reactive oxygen species (ROS) were identified as possible inducers of JNK and CD95L gene transactivation by literature research (Gulow, Kaminski et al. 2005; Guy, Wang et al. 2006; Kiessling, Linke et al. 2010) .
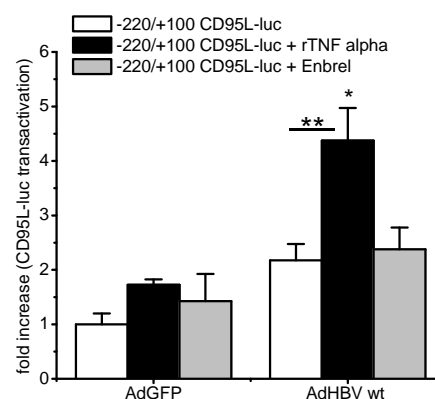

Figure 3.21 CD95L transactivation is not dependent on TNF alpha in HBV-infected hepatocytes
HBV infection leads to the transactivation of the CD95L gene in HepG2 cells. Cells were transiently transfected with 1 µg of the following CD95L promoter construct: -220/+100CD95L-luc. Cells were additionally infected with 10 MOI of AdHBV wt or AdGFP and treated with rTNF-α or the TNF-α inhibitor Enbrel as indicated. Luciferase activity value obtained with AdGFP was set to 1. One representative experiment out of 3 independent experiments is shown, respectively (Mean ± SD, n=3). (*p≤0.01 compared to untreated, **p≤0.01 compared to the next column, as determined by Anova, Bonferroni's multiple range test).

Treatment of AdHBV wt-infected hepatocytes with recombinant TNF-α led to an increased CD95L gene transactivation in HepG2 cells transfected with 1 µg -220/+100 CD95L-luc compared to infection with AdGFP. The treatment with the TNF-α inhibitor Enbrel, however, did not result in a decreased CD95L transactivation in HepG2 cells infected with AdGFP or AdHBV wt (figure 3.21).

To investigate the effect of ROS on the CD95L transactivation, HBV-infected hepatocytes were transfected with 1 μg -220/+100 CD95L-luc and additionally treated with the reactive oxygen species inhibitor N-acetyl L-cysteine (NAC). NAC treatment significantly decreased CD95L-luc transactivation in HBV-infected HepG2 cells (c.f. figure 3.22). This inhibitory effect of NAC on the transactivation of CD95L-luc could also be observed in HepG2 cells treated with bleomycin. This suggests that the CD95L gene is transactivated in HBV-infected hepatocytes through ROS-mediated JNK phosphorylation and subsequent activation of the transcription factor AP-1.
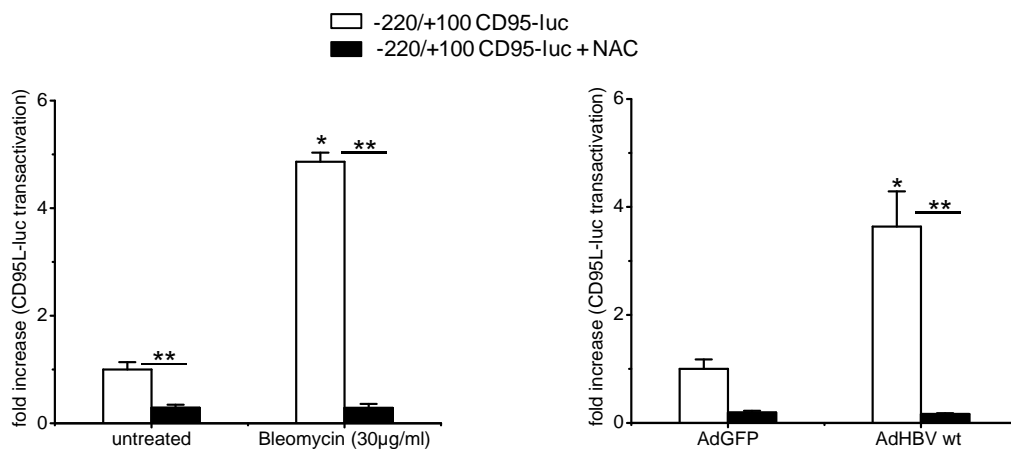

Figure 3.22 CD95L transactivation in HBV-infected HepG2 cells is regulated by reactive oxygen species HBV infection leads to the transactivation of the CD95L gene in HepG2 cells via ROS. Cells were transiently transfected with 1 μg of the following CD95L promoter construct: -220/+100CD95L-luc. Cells were additionally treated with 30 μg/ml bleomycin or infected with 10 MOI of AdGFP or AdHBV wt and treated with the ROS inhibitor N-acetyl L-cysteine (NAC). One representative experiment out of 3 independent experiments is shown, respectively (Mean ± SD, n=3). Luciferase acitivity values obtained with AdGFP were set to 1. (*$p \leq 0.01$ compared to untreated, **$p \leq 0.01$ compared to the next column, as determined by Anova, Bonferroni's multiple range test).

HBV infection triggers the induction of CD95L gene transactivation in a similar manner as it was previously described for chemotherapeutic-induced CD95L transactivation in HepG2 cells. The AP-1 binding site at position +84 - +91 in the 5' UTR of the CD95L gene was identified as the predominant binding site responsible for the transactivation of CD95L upon HBV infection. Furthermore, we could show that HBV infection does not only lead to the transactivation but also to the transcription and finally CD95L expression in the hepatoma cell line HepG2.

3.3 Hepatitis B virus infection and p53 target genes of the intrinsic apoptosis signalling pathway

In this study it was demonstrated so far, that HBV infection triggers the induction of the DDR which in turn leads to the stabilization of p53. This subsequently results in the activation of p53 target genes which are playing a role in viral clearance by the immune system and are important elements of the extrinsic apoptosis signalling pathway. Knowing all this, the question emerges, to which extent the intrinsic apoptotic pathway is involved in the elimination of HBV-infected hepatocytes. In order to investigate the induction of the intrinsic apoptosis signalling pathway, the regulation of prominent members involved in the intrinsic apoptosis pathway upon HBV infection was analyzed.

3.3.1 The p53 targets Bax, Puma and Noxa are activated upon HBV infection

To analyze the expression of the p53 target genes Noxa and Puma in HBV-infected hepatocytes, hepatoma cells were permeabilized, fixed, stained with the corresponding antibody and protein expression was determined by FACSCalibur analysis.

Adenoviral transfer of the different AdHBV constructs and AdGFP as control, resulted in an increased expression of Noxa and Puma in the cytoplasm of HepG2 cells (figure 3.23). Puma expression was slightly higher than the expression of Noxa, with a maximum of 50% Puma positive cells at 48 h post AdHBV wt infection. In contrast, AdHBV wt infection results only in approximately 22% of Noxa positive cells. In account with the data obtained for the extrinsic apoptosis signalling pathway there was almost no effect detectable between AdGFP and AdHBV X- infection. This again highlights the important role of HBx already observed in other experiments of this thesis. The density plots show the distribution of HBV wt infected cells. The X axis present the percentage of GFP positive and therefore AdHBV wt infected cells, while the Y axis indicates the amount of Noxa or Puma positive cells. The upper right (UR) quadrant shows the amount of GFP and Noxa or Puma positive cells.
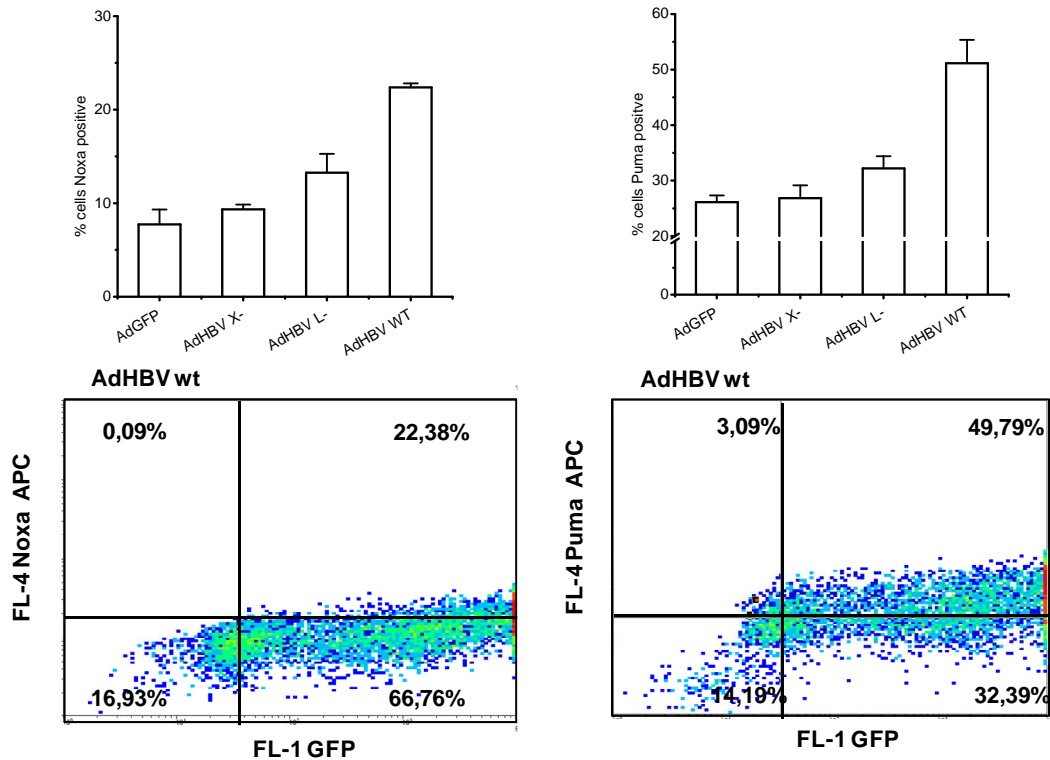
Figure 3.23 HBV infection results in the expression of the p53 targets Noxa and Puma in HepG2 cells

Quantitative flow cytometry analysis (FACSCalibur®) of Noxa and Puma protein expression in HBV-infected HepG2 cells. The upper charts display the fold increase of Noxa or Puma expression upon AdHBV infection. The density plots show the distribution of AdHBV wt infected cells. The X axis present the percentage of GFP positive and therefore AdHBV wt infected cells, while the Y axis indicates the amount of Noxa or Puma positive cells. The upper right (UR) quadrant shows the amount of GFP and Noxa or Puma positive cells. Percent of Noxa or Puma expression was calculated as % Noxa or Puma⁺- % isotype control (Mean ± SD, n=3).

Analogous to the results showing CD95 expression (figure 3.7), adenoviral Adp53 was added in Hep3B cells to increase Noxa and Puma expression (figure 3.24). In contrast to CD95 where Adp53 was needed to obtain an increase in CD95 expression upon HBV infection, AdHBV infection alone was already sufficient to induce a slight increase in Noxa and Puma expression in Hep3B cells. Co-infection of adenoviral p53 and the different AdHBV constructs boosted the effect of AdHBV; however, there was no clear indication for a synergistic effect. Similar to the results obtained in HepG2 cells the constructs containing the viral transcription factor HBx induced Noxa and Puma expression in Hep3B cells, whereas AdGFP and the HBx-lacking AdHBV X- construct did not lead to the expression of both proteins.
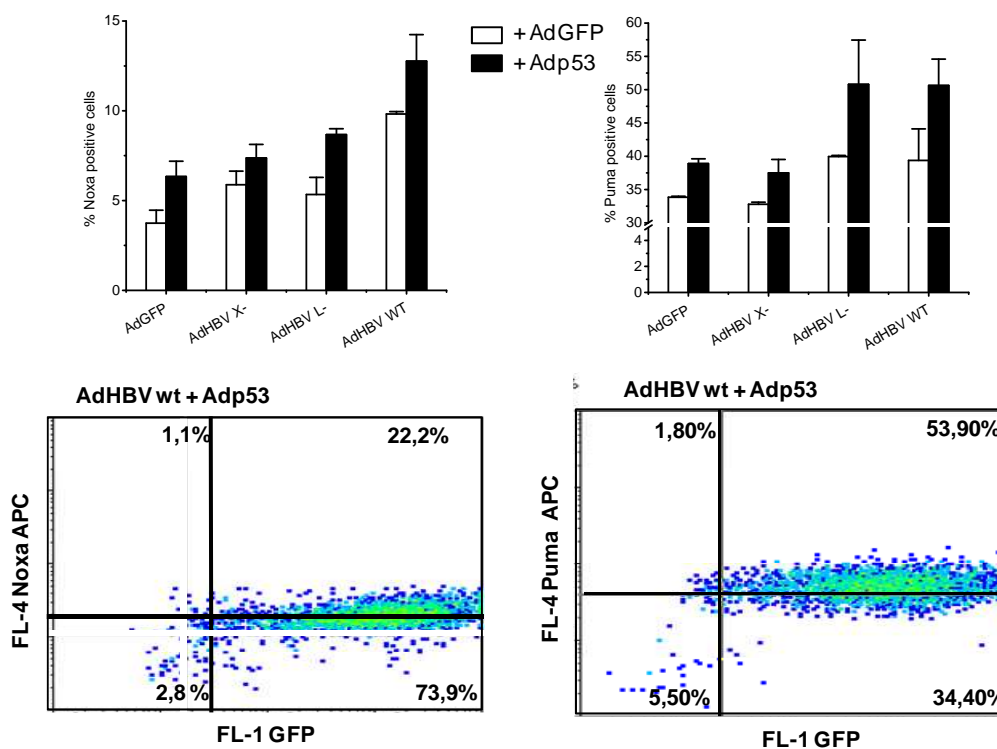
Figure 3.24 HBV infection results in the expression of the p53 targets Noxa and Puma in Hep3B cells

Quantitative flow cytometry analysis (FACSCalibur®) of Noxa and Puma expression in HBV-infected Hep3B cells. The upper charts display the fold increase of Noxa or Puma expression upon HBV infection. To study a possible synergistic effect of Adp53 and AdHBV, Adp53 was co-infected with the adenoviral HBV constructs. The white bars represent the expression of Puma and Noxa induced by AdHBV alone, the black bars show the combined adenoviral infection of Adp53 and the distinct AdHBV constructs. The density plots show the distribution of AdHBV wt infected cells co-infected with Adp53. The X axis present the percentage of GFP positive and therefore AdHBV wt infected cells, while the Y axis indicates the amount of Noxa or Puma positive cells. The upper right (UR) quadrant shows the amount of GFP and Noxa or Puma positive cells. Percent of Noxa or Puma expression was calculated as % Noxa or Puma$^+$- % isotype control (Mean ± SD, n=3).

As a consequence of Noxa and Puma activation upon HBV infection their downstream target Bax was analyzed for transactivation, mRNA transcription and protein expression.

In both, Hep3B and HepG2 hepatoma cell lines, infection with the different AdHBV constructs resulted in an increased Bax gene activity at 24h p.i. In contrast to HepG2 cells (p53$^{+/+}$) where AdHBV infection alone was already sufficient to induce Bax gene transactivation, p53$^{-/-}$ Hep3B cells did not show an increase in Bax gene transactivation at all. Co-infection of Adp53 in Hep3B cells boosted the Bax

transactivation levels. Figure 3.25b shows the statistically significant synergistic effect of Adp53 and the distinct AdHBV constructs.
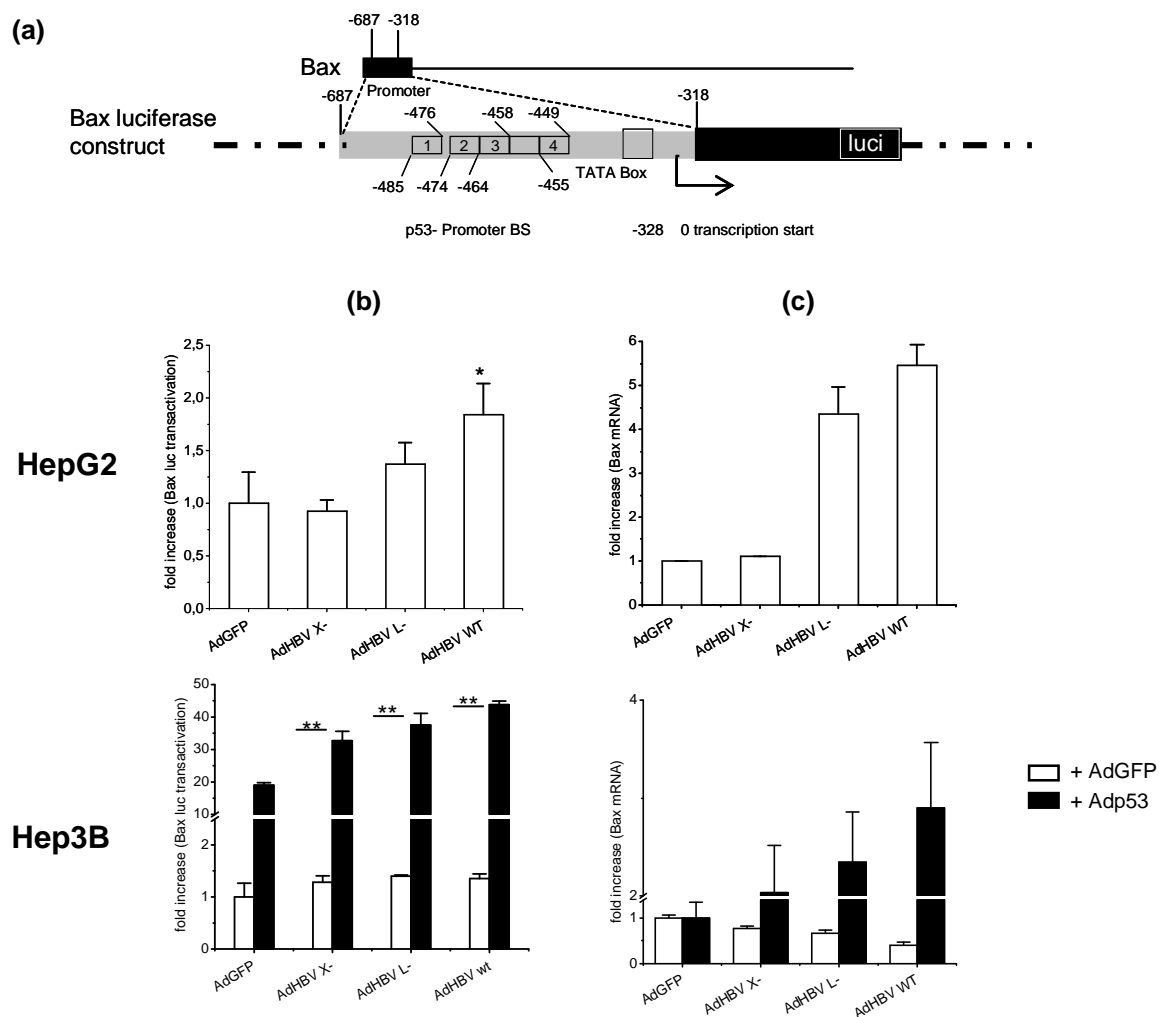

Figure 3.25 HBV infection leads to the transactivation and transcription of the pro-apoptotic Bcl-2 family member Bax HBV infection cooperates with p53 in the transactivation of the Bax gene, leading to an up-regulation of Bax mRNA in HBV-infected hepatocytes. **(a)** Map of the human Bax gene. The intronic p53 binding sites (p53-IBS) are shown. **(b)** Analysis of HBV-dependent Bax luciferase gene activity. HepG2- and Hep3B cells were transfected with 1 µg Bax-Pr/luc reporter plasmid and infected with 10 MOI of the respective adenoviral HBV constructs. Hep3B-cells were additionally infected with Adp53. Shown is the fold increase of each adenoviral HBV construct and AdGFP, calculated relative to the value obtained with AdGFP The differences between AdGFP and AdHBV wt are statistically significant in HepG2 cells (*p≤0.05 compared to AdGFP, **p≤0.001 compared to the next left column as determined by Anova, Bonferroni's multiple range test). **(c)** Quantification of Bax mRNA in HBV-infected hepatocytes by real time PCR 24 h p.i.

One representative experiment out of 3 independent experiments is shown, respectively (Mean ± SD, n=3).

While Adp53 alone induced a 20-fold increase in Bax transactivation, co-infection of Adp53 and the different AdHBV constructs led to a fold increase of 40. In consequence of gene activation, AdHBV infection led to an up-regulation of Bax transcription in HepG2 cells. In contrast and accordingly to the transactivation experiments, no Bax transcription was observed in Hep3B cells. However, after co-infection of AdHBV and exogenous Adp53, Hep3B cells showed similar Bax transcription as observed in HepG2 cells (figure 3.25c). In accordance with the results obtain so far, the induction of Bax transactivation and transcription was less in hepatocytes infected with the HBx negative adenoviral HBV X- construct.
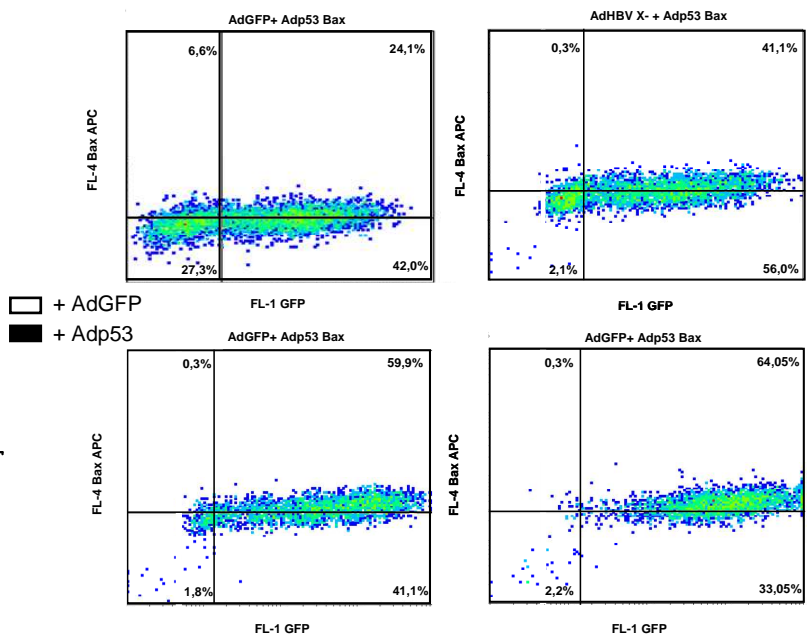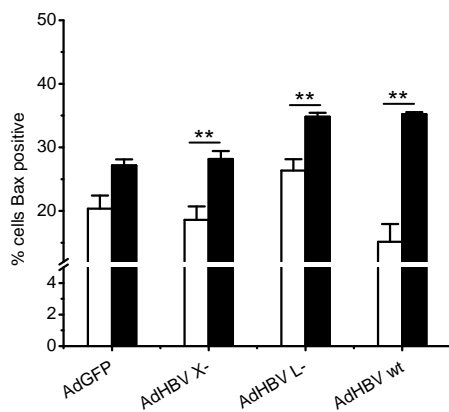
To analyze Bax protein expression in HBV-infected hepatocytes, we used FACSCalibur analysis. Adenoviral transfer of the different AdHBV constructs as well as AdGFP, resulted in an increase in Bax expression in the hepatoma cell line HepG2. The HBx-containing adenoviral HBV constructs, AdHBV L- and AdHBV wt, triggered an increase in the expression of Bax. This was in contrast to the HBx knock-out construct, AdHBV X-. Similar to the results showing CD95 expression (figure 3.7), adenoviral p53 had to be added in Hep3B cells to obtain Bax expression. Co-infection of Adp53 and the described AdHBV constructs resulted in a significant increase in Bax expression highlighting the role of Bax as a p53 target. However, the effect detected was not considered to be synergistic (figure 3.26a and b). The density dot plots demonstrate the distribution of AdHBV-infected hepatocytes with regard to infected, GFP positive, and Bax positive hepatocytes. Lysates of PHHs harvested at 60 h p.i. also clearly indicate the expression of Bax upon HBV infection compared to infection with AdGFP (figure 3.26c).

In summary, HBV infection triggers the activation and expression of p53 target genes of the extrinsic as well as the intrinsic apoptosis signalling pathway. The BH3-only proteins Puma and Noxa as well as the Bcl-2 family member protein Bax could be identified as crucial proteins activated upon HBV infection, thereby contributing to viral clearance.

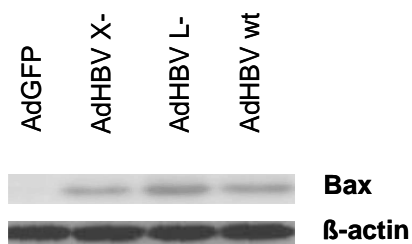**(a)** **HepG2**
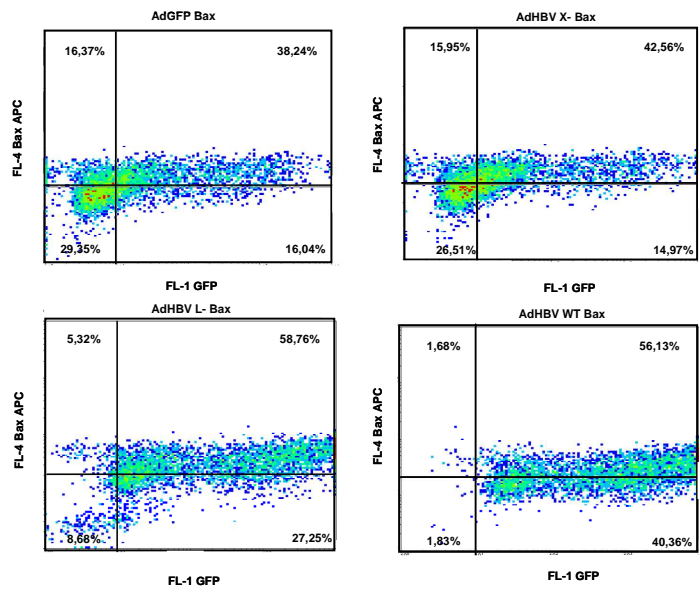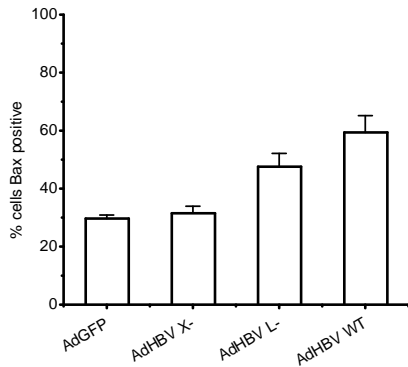
(b) **Hep3B**

(c) **PHH**

Figure 3.26 Quantitative flow cytometry analysis (FACSCalibur®) of Bax protein expression in HBV-infected HepG2- and Hep3B-cells Only HepG2 cells **(a)**, possessing endogenous p53, display up-regulated Bax protein expression. Hep3B **(b)**, lacking p53, were additionally infected with Adp53 to obtain up-regulation of the Bax protein. Percent of Bax expression was calculated as % Bax⁺- % isotype control. Shown is Mean ± SD, n=3. **p≤0.05 compared to the next left column as determined by Anova, Bonferroni's multiple range test. **(c)** Western blot analysis of Bax expression in PHHs. HBV-infected PHHs were lysed 60h p.i. and expression of the Bax protein was investigated. ß- Actin was used as an internal loading control.

3.3.2 The p53-dependent expression of BH3-only and Bcl-2 family member proteins upon HBV infection results in the alteration of mitochondrial outer membrane potential and the subsequent release of cytochrome c

The crucial point in the intrinsic apoptosis signalling pathway is the alteration of the mitochondrial outer membrane potential due to pore formation of the Bcl-2 family members Bax and Bak. Alteration of mitochondrial outer membrane potential results in the release of cytochrome c which forms the apoptosome together with the adapter protein Apaf-1 and pro-caspase 9. In contrast to all the results obtained so far in this thesis, AdHBV infection alone was not sufficient enough to induce a remarkable increase in the mitochondrial outer membrane potential in HepG2 cells. AdHBV wt only led to approximately 5% more cells with altered mitochondrial outer membrane potential than infection with AdGFP in HepG2 cells (figure 3.27). Additional exogenous Adp53 was needed to depict the differences within the adenoviral HBV constructs. According to the results obtained with the mitochondrial proteins Bax, Noxa and Puma infection with Adp53 and AdHBV wt resulted in a significant increase in cells with altered mitochondrial outer membrane potential.
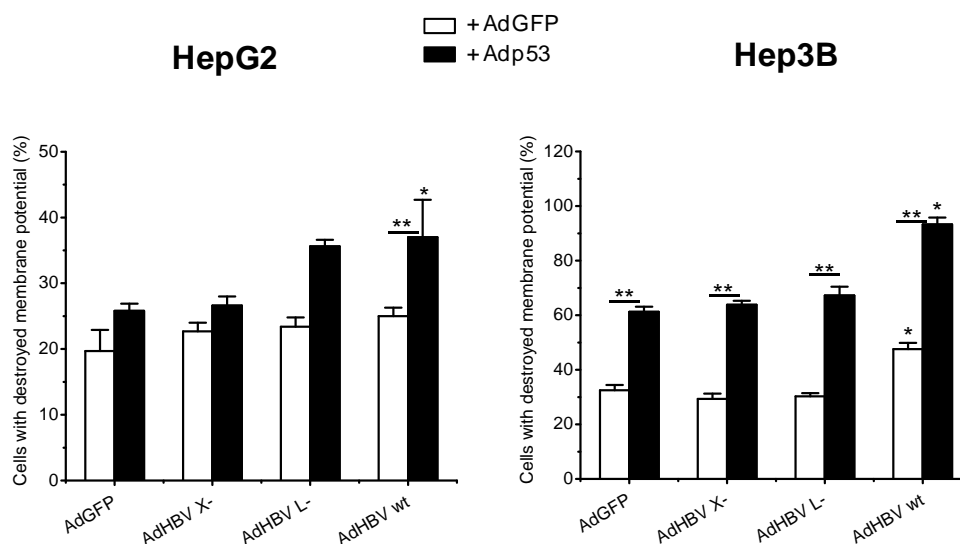
Figure 3.27 HBV infection leads to an alteration of the mitochondrial outer membrane potential FACSCalibur analysis of HepG2- and Hep3B cells following DiIC staining shows an alteration of the mitochondrial membrane potential. Error bars represent standard deviation of 3 independent experiments (Mean± SD, n=3). The difference between AdGFP and Adp53 infected hepatocytes is statistically significant (*p≤0.05 compared to the respective AdGFP, **p≤0.05 compared to the next left column as determined by Anova, Bonferroni's multiple range test).

Quite the reverse was detected in Hep3B cells. In the p53$^{-/-}$ hepatoma cell line AdHBV wt infection alone was already sufficient to induce an alteration of the mitochondrial outer membrane potential. The increase in cells with altered mitochondrial outer membrane potential was significant compared the infection of AdGFP and AdHBV wt. Additional infection with Adp53 boosted the effect of AdHBV significantly compared to AdHBV infection alone. Combined infection of Adp53 and AdHBV wt into Hep3B cells resulted in almost 100% of hepatoma cells with altered membrane potential.

The subsequent release of cytochrome c was also assessed by FACSCalibur analysis (figure 3.28). The cells were treated and stained as already described for the mitochondrial proteins Bax, Puma and Noxa. Again the effect of AdHBV alone and in combination with Adp53 was investigated. The HBx-containing adenoviral HBV constructs reflect the observations of the other functional experiments described in the present thesis. Infection of hepatoma cells with AdHBV L- and AdHBV wt resulted in the elevated release of cytochrome c in HepG2 cells. Additional infection with Adp53 amplified the increase of cytochrome c positive HepG2 and Hep3B cells.
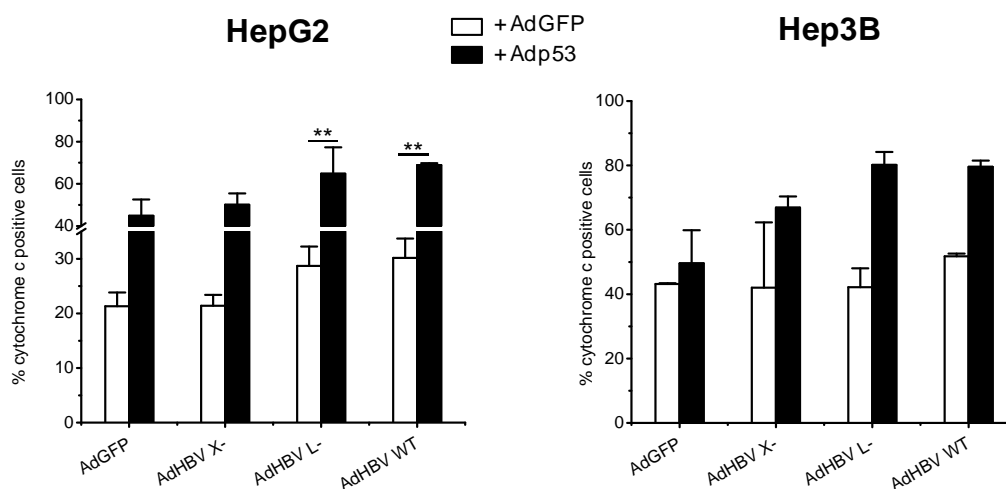
Figure 3.28 Alteration of the mitochondrial outer membrane potential upon HBV infection triggers the release of cytochrome c FACSCalibur analysis of HepG2- and Hep3B cells following intracellular cytochrome c staining. Cytochrome c release is displayed in the amount of cytochrome c positive hepatocytes. Error bars represent standard deviation of 3 independent experiments (Mean± SD, n=3). The difference in the case of AdHBV L- and AdHBV wt infected hepatocytes co-infected with Adp53 and AdGFP are statistically significant (**p≤0.05 compared to the next left column as determined by Anova, Bonferroni's multiple range test).

In summary, hepatitis B Virus infection triggers the activation of the DNA damage response leading to the stabilization and activation of the tumour suppressor p53. Activation of p53 upon HBV infection induces the activation of different p53 target genes playing important roles in the activation of the extrinsic as well as the intrinsic apoptosis signalling pathway. The expression of proteins involved in apoptosis signalling leads to the alteration of the mitochondrial membrane potential, the subsequent release of cytochrome c and finally to the induction of apoptosis in HBV-infected hepatocytes and viral clearance. Our results propose a new p53-dependent model for virus elimination and show that p53 beyond its role as a tumour suppressor also plays an important role in viral clearance.

4. Discussion

Chronic hepatitis B virus infection is a major risk factor for the development of hepatocellular carcinoma and more than 350 million patients are chronically infected with HBV worldwide. Therefore, HBV represents a foremost health burden especially in developing countries (El-Serag 2011). HBV infection is known to induce acute and chronic necroinflammatory liver injury, death of hepatocytes and to promote hepatocarcinogenesis resulting in the development of HCC, the most common primary malignant liver cancer and the third leading cause of cancer-related death in the world. It is still not clear whether the virus itself acts as a tumour initiator or if the subsequent inflammation, causing liver regeneration and cirrhosis, acts as a tumour promoter in hepatocarcinogenesis (Hussain, Schwank et al. 2007).

The partially double-stranded hepatitis B virus is acting as a stealth virus and is, therefore, hardly detected by the innate immune system. Due to this reason, the immune system is unable to efficiently clear HBV from the liver leading to persistence of virus infection (Guidotti and Chisari 2006). Although, it is well known that mainly CD8^{+} T cells are involved in the immune response against HBV, the precise molecular interactions between the immune system and HBV-infected hepatocytes leading to virus elimination are not yet completely understood (Chisari and Ferrari 1995; Protzer and Schaller 2000). The recent findings from Arzberger et al. (Arzberger, Hosel et al. 2010) demonstrating that apoptosis of HBV-infected hepatocytes results in the release of non-infectious viral particles, however, provide evidence for the important role of apoptosis in the elimination of HBV-infected hepatocytes. The precise steps in the process leading to the induction of apoptosis in virus-infected hepatocytes and the signalling pathways up-regulated in infected host cells to combat the virus are still awaiting elucidation.

The data obtained in the present study allow us to propose a new p53-dependent model for the elimination of HBV-infected hepatocytes (c.f. figure 4.1). We could show that p53 beyond its role as a tumour suppressor plays an essential role in viral defence. HBV infection results in the activation of the DNA damage response (DDR) and the subsequent phosphorylation of p53 in an ATM/ATR-dependent manner. Stabilization of p53, due to serine 15 phosphorylation leads to the activation of prominent members of the extrinsic and intrinsic apoptosis signalling pathway. The activation of both, p53 target genes of the extrinsic and of the intrinsic apoptosis

signalling pathway, upon HBV infection suggests an interaction between HBV-induced p53 stabilization and apoptosis induction. We identified a functional CD95 pathway, a classical p53 downstream target, to be essential for the elimination of HBV-infected hepatocytes. However, not only CD95 is activated upon HBV infection in a p53-dependent manner, Bax, Puma and Noxa, whose genes are also up-regulated by p53, were also identified as crucial proteins involved in the elimination of HBV-infected hepatocytes. The expression of proteins involved in apoptosis signalling leads to the alteration of the mitochondrial outer membrane potential, the subsequent release of cytochrome c and finally the induction of apoptosis in HBV-infected hepatocytes, which is accompanied by the cleavage of PARP-1. Of note, experiments with primary human hepatocytes (PHH) confirmed the results we obtained in hepatoma cell lines and point towards the clinical relevance of our findings. In addition, our model provides evidence for the pro-apoptotic role of the viral HBx protein. Infection of the hepatoma cell lines and PHHs with the HBx knock-out adenoviral HBV construct, demonstrate the important role of HBx in the induction of apoptosis. We could show that infection with the HBx knock-out construct resulted in a decreased phosphorylation of proteins involved in the DNA damage response and, therefore, also in a minor activation of the p53 targets, shown to be involved in the elimination of HBV-infected hepatocytes. We, thus, hypothesise that both, DDR-induced stabilization of p53 and the activation of proteins involved in the apoptosis machinery, contribute to HBx-dependent apoptosis induction.

Our data suggest a new p53-dependent model for virus elimination and show that p53 beyond its role as a tumour suppressor plays an important role in viral clearance. In summary, we postulate, that an intact p53 status and a functional CD95 apoptosis signalling pathway are essential for the elimination of HBV-infected hepatocytes by sensitizing them towards apoptosis, thus preventing chronic hepatitis B virus manifestation. The identification of prominent members of the apoptosis signalling pathways as crucial proteins activated upon HBV infection demonstrate an underlying mechanism for the diverse outcomes (clearance *vs.* chronicity) of an HBV infection. Furthermore, the significant contribution of p53 in cellular antiviral defence suggests a potential implication in the pathogenesis of HBV infection and, thus, opens new therapeutic options.
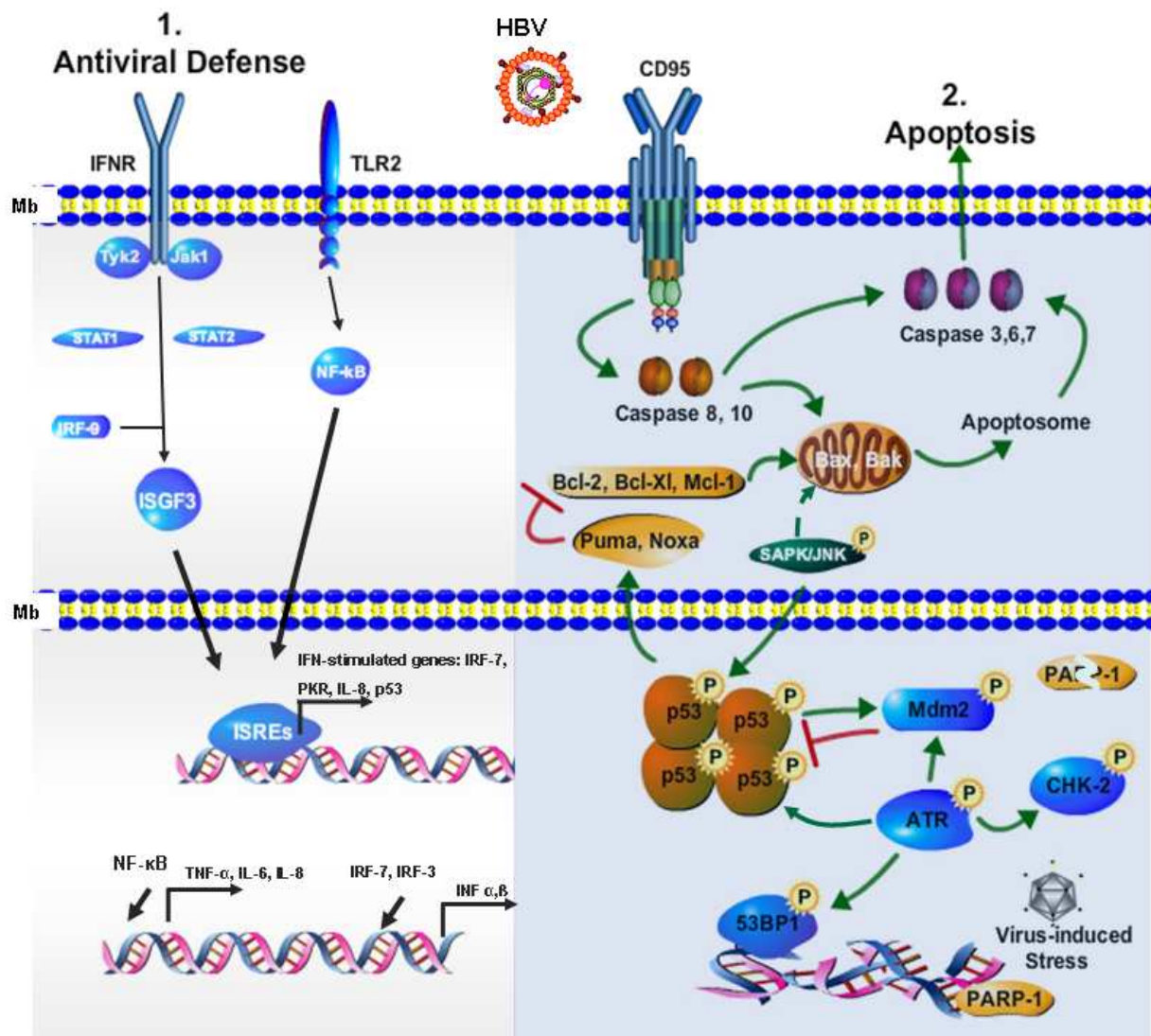
Figure 4.1 Key steps in the elimination of hepatitis B Virus infected hepatocytes HBV infection leads to the induction of a weak interferon response and, therefore, the activation of the innate immune response (1. displayed in grey on the left side). More importantly, the tumour-suppressor p53 is activated by phosphorylation on serine 15. Upon phosphorylation, p53 induces the initiation of the intrinsic and extrinsic apoptosis signalling pathway by up-regulating the BH3-only proteins Puma and Noxa, which in turn activate Bax and Bak and induce mitochondrial membrane permeabilization (2. displayed in blue on the right side). Up-regulation of CD95 results in an increased responsiveness towards CD95-mediated apoptosis of HBV-infected hepatocytes. Binding of the CD95L (CD95L is not depicted in the figure) to its corresponding receptor results in receptor clustering and the recruitment of FADD (not depicted in the figure) as well as the initiator caspases 8 and 10. DISC (not labelled in the figure) formation initiates the activation of the apical caspases, driving their autocatalytic processing and the release into the cytoplasm, where they activate the effector caspases 3, 6 and 7. Finally, the activation of the death pathways leads to the induction of apoptosis and, therefore, elimination of HBV-infected hepatocytes. An additional mechanism of p53 stabilization is its phosphorylation by JNK, which also promotes apoptosis by inducing the translocation of Bax to the mitochondria.

Pathway was generated using the pathway builder from www.proteinlounge.com.

Intriguingly, HBV infection also leads to the phosphorylation of the stress-activated c-Jun N-terminal kinase (JNK), known to trigger serine 15 phosphorylation of p53, thereby abolishing its association with Mdm2 (Fuchs, Adler et al. 1998). Furthermore, JNK is able to phosphorylate c-Jun which is a component of the transcription factor AP-1 (Fuchs, Adler et al. 1998). In the present study we show that HBV-induced phosphorylation of JNK triggers CD95L gene transactivation in an AP-1-dependent manner which leads to the, so far, unrecognized capability of HBV-infected hepatocytes to secrete CD95L by themselves (c.f. figure 4.2). Thus, our model provides evidence that HBV infection leads to the transactivation, transcription and expression of membrane-bound and soluble CD95L. The 5' UTR AP-1 binding site within the CD95L gene was identified as the predominant transcription factor binding site responsible for CD95L transactivation upon HBV infection. Furthermore, we could show that reactive oxygen species (ROS) are necessary for the transactivation of the CD95L gene in HBV-infected hepatocytes.

In summary, our model indicates that HBV infection leads to JNK-mediated AP-1-dependent transactivation, transcription and expression of soluble CD95L, proposing a new mechanism for the elimination of HBV-infected hepatocytes independently of cytotoxic T cells.
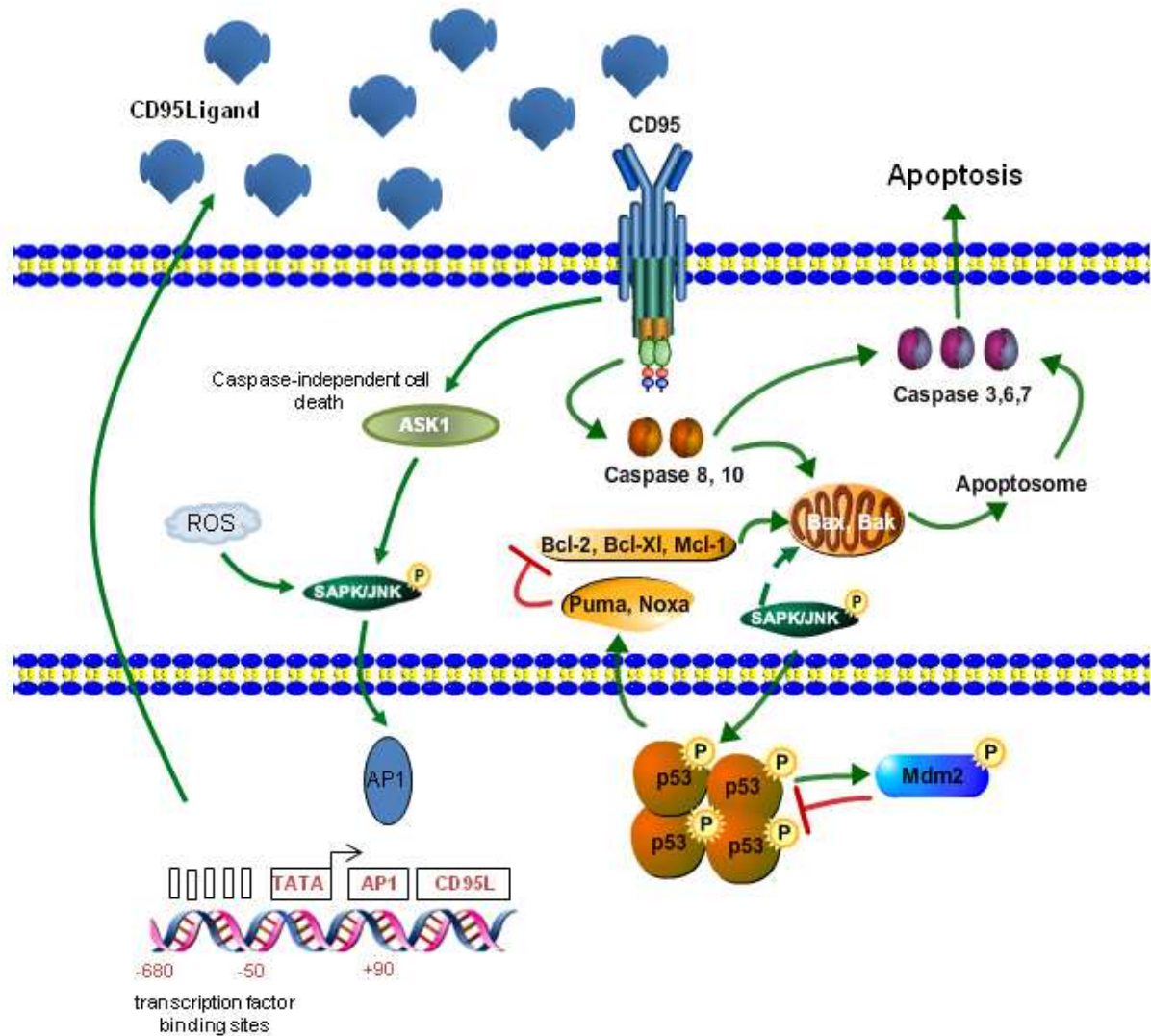
Figure 4.2 The AP-1 element in the 5' UTR of the CD95L gene is responsible for CD95L expression in HBV-infected hepatocytes HBV infection results in the phosphorylation of the tumour suppressor p53 and the stress activated c-Jun N-terminal kinase (JNK) (c.f. figure 4.1). The activation of p53 and JNK results on the one hand in the activation of the extrinsic and intrinsic apoptosis signalling pathway and on the other hand in the JNK-mediated activation of the transcription factor AP-1. Activated AP-1 binds to the 5' UTR AP-1 binding site of the CD95L gene which results in the subsequent transcription and expression of sCD95L. The secretion of sCD95L upon HBV infection can trigger CD95-mediated apoptosis in other HBV-infected cells or on the ligand secreting hepatocytes themselves. Beside the activation of the caspase-dependent death pathway, the CD95L-CD95 complex can also trigger caspase-independent cell death by ASK1 (Apoptosis signal-regulating kinase 1)-dependent JNK phosphorylation and the subsequent accumulation of Bax at the mitochondria. Pathway was generated using the pathway builder from www.proteinlounge.com.

4.1 An intact p53 status is required for the elimination of HBV-infected hepatocytes

Findings obtained in the present study allow us to propose that an intact p53 status plays a crucial role in the outcome of an acute HBV infection (viral clearance *vs.* chronicity). In line with Webster et al. (Webster and Bertoletti 2002) who argued that HBV might be able to induce apoptosis of its host cells even though it is classified as a non-cytopathic virus, we show that p53 stabilization sensitizes hepatitis B virus-infected host cells towards CD95-mediated apoptosis. That apoptosis is a powerful antiviral defence mechanism to erase HBV infection as HBV particles which are released from apoptotic hepatocytes were immature, non-enveloped capsids and were proved not to be infectious has been shown recently (Arzberger, Hosel et al. 2010). However, the direct molecular mechanisms leading to apoptosis and, therefore, elimination of HBV-infected cells have so far been controversially described.

While Terradillos et al. showed in transgenic mice that functionally intact p53 was not required to transduce the death signal (Terradillos, Pollicino et al. 1998), instead they demonstrated HBx to be the essential protein, our data provide a new, p53-dependent model for the elimination of HBV-infected hepatocytes and, therefore, prevention of chronicity. Here we show that p53 and its target CD95 are essential for the induction of apoptosis in HBV-infected hepatocytes and their elimination (c.f. figure 3.4, 3.8 and 3.12). Another protein found to play a controversial role in the induction of apoptosis is the viral HBx. Dependent on the differentiation status of hepatocytes HBx can promote or inhibit apoptosis (Pollicino, Terradillos et al. 1998; Pan, Duan et al. 2001; Kim and Seong 2003; Arzberger, Hosel et al. 2010; Hsieh, Hsu et al. 2011). Taking the controversial role of HBx in the induction of apoptosis of HBV-infected hepatocytes, our data provide evidence for the pro-apoptotic role of HBx. This is in accordance with the findings described by Pollicino, Kim and Hu (Pollicino, Terradillos et al. 1998; Kim and Seong 2003; Liu and Wu 2010; Hu, Chen et al. 2011). While Pan et al. described that HBx via NFκB protects against CD95-mediated apoptosis in HepG2.2.15 cells (stably transfected with the complete HBV genome) and cells infected with HBxAg-containing recombinant retroviruses (Pan, Duan et al. 2001), we show that HBx is needed for the up-regulation of CD95 and, therefore, the responsiveness of HBV-infected hepatocytes towards CD95-mediated apoptosis. In contrast to the systems used by Pan et al. our model allows us to work

in an experimental setup simulating the *in vivo* conditions. By the use of different adenoviral vectors (containing either the whole HBV genome or specific knock-outs of designated viral proteins; AdHBV wt, AdHBV L- and AdHBV X-) and two different hepatoma cell lines and, of note, the use of primary human hepatocytes we could prove the important role of HBx in the up-regulation of CD95 in HBV-infected hepatocytes.

Increased p53 activity during HBV infection in HepG2 cells and PHH leading to CD95-mediated and HBx-dependent apoptosis directs to the conclusion that p53 is a commonly activated protein in host cells during HBV infection. This is in accordance with Chirillo et al. who found a connection between HBx and p53-mediated apoptosis in mouse fibroblasts transfected with a human HBx-containing dexamethasone inducible mouse mammary tumor virus (MMTV)-X expression vector (Chirillo, Pagano et al. 1997).

Previous studies showed an interaction between type 1 interferons (INF-α/β), as the first line of antiviral defence, and the transcriptional up-regulation of p53. The presence of an active ISRE (Interferon-stimulated response element) in the promoter of p53 leads to its INF-α/β-mediated transcription (Takaoka, Hayakawa et al. 2003; Vilcek 2003). p53, as a type 1 IFN signalling downstream target, also contributes to an IFN-inducing positive feedback loop, established through the p53-dependent transcriptional induction of IFN regulatory factor 9 (IRF9), which is a part of the IFN-stimulated gene factor 3 (ISGF3) (Munoz-Fontela, Macip et al. 2008). However, type 1 interferons rather seem to be responsible for p53 accumulation than for activation, but increased p53 concentrations can enhance cellular responses to stress signals that activate p53 (Vilcek 2003). Munoz-Fontela et al. (Munoz-Fontela, Macip et al. 2008) propose a two phase model for the different roles of p53 in antiviral defence. First, p53 is enhancing IFN- production and signalling to counteract viral infection. In a second step, p53 inhibits viral propagation by the elimination of infected cells through the induction of apoptosis (Munoz-Fontela, Macip et al. 2008).

Initial recognition of HBV infection and the induction of type 1 interferon response may be triggered by toll-like receptors (Boehme and Compton 2004; Chang and Lewin 2007). Furthermore, it has been reported that HBV replication elicits a type 1 interferon response (Lucifora, Durantel et al. 2010). Although, type 1 interferons are able to inhibit transcriptional and posttranslational steps in HBV replication, HBV

infection, in contrast to most viral infections does not seem to induce a strong IFN type 1 response. Instead, it has been reported that HBx is able to inhibit type 1 interferon induction (Jiang and Tang 2010). Therefore, HBV is considered as being a "partially stealth" virus (Wieland and Chisari 2005; Bauer, Sprinzl et al. 2011). Taking the controversial issues into consideration, type 1 interferon seems to be not the only player in viral defence against HBV.

In the present study we could show that in contrast to other viruses, p53 accumulation after HBV infection is not type 1 interferon-mediated. Our results indicate no induction of p53 gene transcription upon HBV infection (results not shown), instead our findings specify that p53 is stabilized and activated by Ser-15 phosphorylation like in a classical DNA damage response (c.f. figure 3.2).

In this study we demonstrate that HBV infection leads to the activation of proteins involved in the DDR, which directs to the conclusion that HBV infection leads to cellular and replication stress, due to the persistent presence of exogenous material and, therefore, to the induction of the DNA damage response. In both hepatoma cell lines and, of note, in primary human hepatocytes, the phosphorylation of ATR and CHK2 on the amino acid residues Ser^{428} and Thr^{68}, respectively as well as 53BP1 accumulation could be observed, indicating the activation of a DDR (c.f. figure 3.2 and 3.3). The main role of the DDR is the protection of the genomic integrity against environmental or spontaneous DNA lesions, occurring during normal DNA metabolism. The activation of the DDR counteracts the induced DNA damage by the activation of specific and different DNA repair mechanisms (Ciccia and Elledge 2010). The outcome of the DDR ranges from cell cycle arrest and DNA repair to the induction of cell death. In the case of severe DNA damage selective "killing" helps to protect the genetic integrity, therefore, apoptosis is a frequently used pathway. The phosphorylation of p53 on serine 15, a typical phosphorylation site upon DNA damage (Wang 2001), demonstrates the important link between DNA damage and apoptosis in HBV-infected hepatocytes (c.f. figure 3.2). The important role of ATM/ATR-dependent p53 phosphorylation in the induction of apoptosis upon HBV infection was confirmed by the use caffeine, an ATR/ATM inhibitor. The decrease of apoptosis after treatment of HBV-infected hepatocytes with caffeine strengthened the hypothesis that DNA damage-induced p53 phosphorylation triggers the induction of apoptosis in HBV-infected hepatocytes (c.f. figure 3.5). Bearing in mind that apoptosis is the last choice; HBV infection must trigger severe DNA damage or

cellular stress to induce apoptosis rather than cell cycle arrest. The generation of cccDNA in the nucleus of infected hepatocytes could indeed trigger replication stress, induced by unscheduled replication of the cccDNA for virus propagation. Furthermore, HBV genome integration has been observed in patients suffering from chronic HBV infection (Shafritz, Shouval et al. 1981). Both, unscheduled replication as well as genomic integration causing genomic instability could generate DNA lesions leading to the activation of the DDR.

In contrast to Zhao et al. (Zhao, Hou et al. 2008), who described that the ATR-dependent p53 stabilization in hepatocytes inoculated with HBV-positive serum has no consequences for the virus, due to viral degradation of the proteins involved in the DNA damage response, the present study clearly describes the consequences of ATR-dependent p53 phosphorylation for the virus. In the HBV-infected hepatoma cell lines, HepG2 and Hep3B, p53 phosphorylation leads not only to the stabilization of p53 but also to the activation of several p53 target genes involved in the extrinsic and intrinsic apoptosis pathway and finally to the induction of apoptosis. Of note, ATM/ATR-dependent p53 phosphorylation and the following activation of p53 target genes was also detected in PHHs which demonstrates the clinically relevance of our findings. Figure 3.4 provides evidence that HBV-induced cellular stress, the subsequent activation of the DNA damage response and the activation of p53 target genes involved in the apoptosis machinery is sufficient enough to induce apoptosis.

The fact that AdHBV L- infection did not lead to the induction of apoptosis in p53$^{-/-}$ Hep3B cells supports the suggestion that p53 is a crucial protein involved in the elimination of HBV-infected hepatocytes. However, the AdHBV wt construct was able to induce apoptosis in the absence of p53 (c.f. figure 3.4). Since HBV wt induces the highest activation of proteins involved in the DDR (c.f. figure 3.2) and, therefore, probably the most extended damage, it is possible that the role of p53 in Hep3B cells is taken over by another p53 family member, p73, a transcription factor also possessing pro-apoptotic functions, in the case of p53 damage or absence. Transfection of the p53 hot-spot mutant, p53R248W, decreases apoptosis induction in HBV-infected hepatocytes compared to HBV-infected hepatocytes transfected with a control plasmid (c.f. figure 3.12. c). Similar to the results obtained in Hep3B cells with HBV infection alone, the p53 hot-spot mutant p53R248W did not completely inhibit the apoptosis of HBV-infected hepatocytes. Therefore, the possibility that the

p53 family member p73 is able to substitute for mutant p53 might be an important implication for future therapeutic options for the treatment of chronic HBV infection.The role of p73 in the induction of apoptosis, tumourigenesis and tumour response to therapy was described in detail by Müller et al. (Müller, Schleithoff et al. 2006).
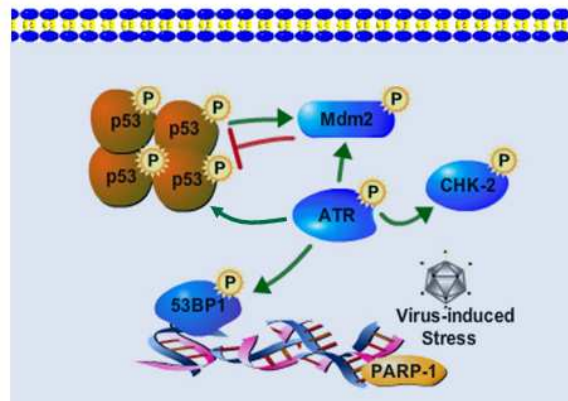

Figure 4.3 HBV infection triggers the phosphorylation of p53 through the DNA damage response Virus infection results in unscheduled replication and/or genomic integration of HBV into the host genome leading to cellular stress and, therefore, the onset of the DNA damage response. The phosphorylation of ATR on serine 428, the activation of CHK2 due to its phosphorylation on threonine 68 and the accumulation of 53BP1 are reliable indicators of DNA damage. Serine 15 phosphorylation of p53 follows the phosphorylation of proteins involved in DNA damage and abolishes its association with its negative regulator Mdm2 (not shown here). Pathway was generated using the pathway builder from www.proteinlounge.com.

4.2 HBV infection and the CD95 death receptor pathway

An important pro-apoptotic gene transactivated by p53 is the death receptor CD95, which was found to play an important role in the elimination of HBV-infected hepatocytes and the induction of the immune response in an *in vivo* experiment performed in our group, recently. Briefly, CD95-deficient mice (B6.MRL-*Fas*^{lpr}/J) and C57BL/6 mice were infected with AdHBV wt and monitored for 28 days. While C57BL/6 mice were able to control HBV infection by inducing apoptosis in HBV-infected hepatocytes and recovered within 28 days, B6.MRL-*Fas*^{lpr}/J mice could not eliminate the virus. C57BL/6 mice showed signs of seroconversion after day 14 indicating the onset of an immune response against HBV, whereas there was no antiHBs detectable in B6.MRL-*Fas*^{lpr}/J mice. Hence, we postulated, that an intact

CD95 apoptosis signalling pathway is essential for the elimination of HBV-infected hepatocytes (unpublished data).

In vitro analysis performed in hepatoma cell lines and, of note, also in PHHs infected with HBV allowed us to further analyse the important role of CD95 in the elimination of HBV-infected hepatocytes and to investigate the molecular mechanism involved in the CD95-mediated elimination of HBV-infected host cells. HBV infection led to the transactivation, transcription and expression of CD95 in hepatoma cell lines (c.f. figure 3.6). The time-dependent CD95 transactivation upon HBV infection has been shown by a former MD student, Andres Rademacher (unpublished data). Since p53 enhances not only the gene activation (Müller, Wilder et al. 1998) but also the expression of CD95, a direct interaction between the accumulation of p53 and the expression of CD95 on the surface of HBV-infected cells is suggested. In accordance with the *in vivo* results the importance of CD95-mediated apoptosis in viral clearance is demonstrated in this thesis. Since CD95-deficient mice (B6.MRL-*Fas*^lpr/J) were not able to eliminate the virus, we postulate that the predominant extrinsic apoptotic pathway involved in HBV clearance is the CD95 pathway. The important role of an intact p53 status for the activation of the CD95 pathway in HBV-infected hepatocytes was demonstrated by the use of the p53 hot-spot mutant p53R248W. This mutant did not only reduce the induction of apoptosis in hepatocytes infected with HBV, it also reduced CD95 transactivation and receptor expression on the cell surface of Hep3B cells (c.f. figure 3.12). Therefore, the data obtained with the p53 hot-spot mutant further contribute to the model, which suggests that inhibition of the CD95-mediated apoptosis of HBV-infected hepatocytes leads to viral persistence. This suggests a possible synergy of alterations of the p53 pathway and HBV-induced hepatocarcinogenesis.

Stimulation and blocking experiments of the CD95 death receptor system revealed that HBV infection triggers CD95 receptor expression and consequently sensitizes HBV-infected hepatocytes towards CD95-mediated apoptosis (c.f. 3.7 and 3.8). While the agonistic antibody anti-APO-1 increased CD95-mediated apoptosis in HepG2 cells significantly, the necessary addition of exogenous p53 into Hep3B cells to actually obtain CD95 expression already resulted in up to 90% apoptosis in combination with AdHBV wt. Therefore, the addition of anti-APO-1 could not further enhance CD95-mediated apoptosis induction in Hep3B cells. However, the important role of CD95 could be demonstrated more clearly upon treatment of HBV-infected

cells with the CD95L containing supernatant of Jurkat 16 cells (SNJ16). In both cell lines SNJ16 treatment resulted in CD95-mediated apoptosis induction, which was confirmed by the use of the CD95L inhibitor APG101 (CD95Fc), a decoy receptor for CD95L.
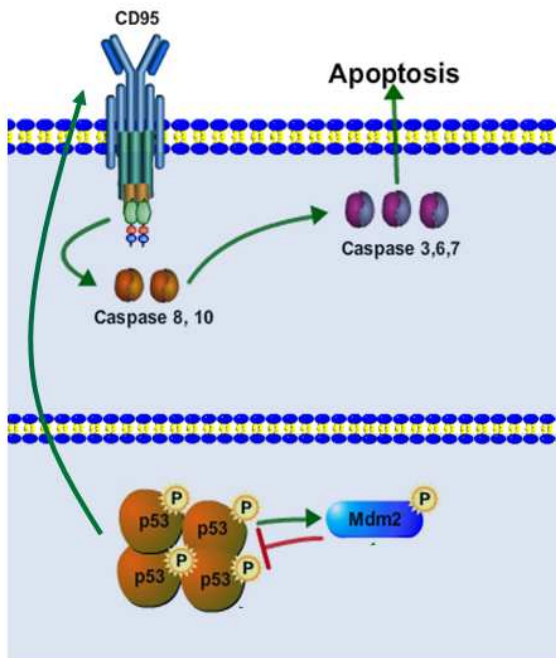

Figure 4.4 HBV infection leads to the transactivation, transcription and expression of functional CD95 Virus-induced p53 stabilization results in the transactivation of the p53 target gene CD95 and the following expression of functional CD95 on the surface of HBV-infected hepatocytes sensitizing the cells towards CD95-mediated apoptosis. Pathway was generated using the pathway builder from www.proteinlounge.com.

Another death receptor, TRAILR2, has also been described to be associated with HBV infection (Liang, Liu et al. 2007). This is in accordance with our results showing an increased TRAILR2 transcription and expression upon HBV infection (c.f. figure 3.11), but according to our results obtained in the present study (*in vivo* and *in vitro*) TRAILR2 seems to play a rather subordinate role in the elimination of HBV-infected hepatocytes.

4.3 Caspase-independent induction of apoptosis in HBV-infected hepatocytes

The fact that treatment with APG101 did not completely protect HBV-infected hepatocytes from apoptosis, leads to the hypothesis that the CD95 pathway is indeed the predominant but not the only mechanism resulting in the apoptosis of HBV-infected hepatocytes (c.f. figure 3.8). A small amount of HBV-infected hepatocytes must be eliminated through mechanisms independent of CD95. Regarding the fact that HBV infection did not only lead to the phosphorylation and stabilization of p53 but also of JNK, the role of JNK in apoptosis induction of HBV-infected hepatocytes

should not be disregarded. As a member of the MAPK superfamily, JNK is able to phosphorylate several transcription factors including c-Jun and p53 as well as non-transcription factors such as members of the Bcl-2 protein family (Liu and Lin 2005). The role of JNK in the induction of apoptosis is, so far, well-described, proposing that JNK activation can have pro-apoptotic or anti-apoptotic functions (Lin and Dibling 2002; Yu, Minemoto et al. 2004; Liu and Lin 2005). HBV-induced phosphorylation of JNK favours the pro-apoptotic function of JNK, as HBV infection results in the induction of apoptosis. The Bax subfamily of Bcl-2-related proteins plays an essential role in JNK-mediated apoptosis. It has been shown that JNK promotes the translocation of the Bcl-2 protein family member Bax to the mitochondria triggering cytochrome c release (Tsuruta, Sunayama et al. 2004).

In this study we demonstrate that HBV infection elicits the activation of Bax as well as the subsequent release of cytochrome c (c.f. figure 3.26). The up-regulation of the p53 target genes Noxa, Puma and Bax upon HBV infection and the boosted activation after exogenous Adp53 addition show a connection between p53 stabilization and the activation of the intrinsic apoptosis signalling pathway upon HBV infection. Of note, accumulation of Bax could also been shown in primary human hepatocytes. The consequent alteration of the mitochondrial membrane potential and the release of cytochrome c, due to the binding of Bax and Bak to the voltage-dependent anion channel (VDAC) (Tsuruta, Sunayama et al. 2004) are clear hints for the activation of the intrinsic apoptosis signalling pathway and the induction of apoptosis.

Another mechanism leading to the alteration and the loss of mitochondrial membrane potential is the localization of the hepatitis B virus protein HBx, which is predominantly found in the cytoplasm close to the mitochondria. Laser confocal microscopy has shown that HBx directly interacts with the outer mitochondrial voltage-dependent anion channel VDAC3 leading to a decrease in the mitochondrial membrane potential. The HBx-induced loss of the mitochondrial outer membrane potential (MOMP) has been linked to cell death (Waris, Huh et al. 2001; Shirakata and Koike 2003; Lim, Kwon et al. 2010). The p53-independent alteration of the MOMP and the following cytochrome c release induced directly by HBx is another possible pathway explaining the significantly increased alteration of MOMP and the increased apoptosis induction in p53$^{-/-}$ Hep3B cells infected with HBV, independent of the extrinsic CD95-mediated apoptosis pathway.
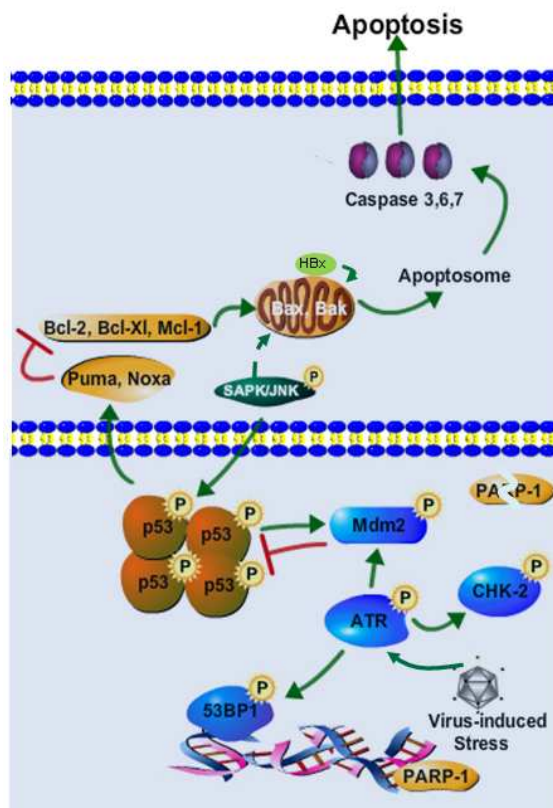
Figure 4.5 Caspase-independent apoptosis induction in HBV-infected hepatocytes Stabilization of p53 leads to the activation of the p53 targets Puma, Noxa and Bax. Activation of Noxa and Puma inhibit the function of the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-X$_l$ and Mcl-1 leading to the activation of the pro-apoptotic protein Bax. Bax binding to the voltage-dependent anion channel (VDAC) results in a decrease of the mitochondrial membrane potential, the subsequent release of cytochrome c and finally the induction of apoptosis. JNK-mediated translocation of the Bcl-2 protein family member Bax to the mitochondria is also triggering cytochrome c release. The localization of HBx at the mitochondrial membrane and its direct interaction with the outer mitochondrial voltage-dependent anion channel VDAC3 is additionally leading to a decrease in the mitochondrial membrane potential. Pathway was generated using the pathway builder from www.proteinlounge.com.

The role of the tumour suppressor p53, the DNA damage response and the extrinsic and intrinsic apoptosis signalling pathway in the elimination of HBV-infected hepatocytes are described and discussed in detail in the present study. It is shown that the observed p53 stabilization and the subsequent activation of the apoptosis machinery in HBV-infected hepatocytes are dependent on the activation of the DNA damage response.

4.4 The role of the viral HBx protein in the elimination of HBV-infected cells

The present data demonstrate the important role of HBx in the elimination of HBV-infected hepatocytes; therefore, providing evidence for the pro-apoptotic function of HBx. In the present study we could describe the role of HBx in the induction of caspase-dependent and caspase-independent cell death. This is in accordance with Lim et al. (Lim, Kwon et al. 2010) who demonstrated the caspase-independent role of HBx in the induction of cell death. In contrast to the HBx knock-out HBV construct AdHBV X-, the HBx-containing constructs, AdHBV L- and AdHBV wt, were found to be able to induce CD95 receptor up-regulation and the activation of the pro-apoptotic Bcl-2 family members Bax, Puma and Noxa. Furthermore, only the infection with HBx-containing adenoviral constructs resulted in an adequate phosphorylation of prominent members of the DNA damage response. The marginal induction of a proper DNA damage response caused by HBx-negative constructs compared to infection with AdHBV wt and the consequent weaker p53 accumulation and stabilization are possible explanations for the reduced expression of CD95 and the pro-apoptotic Bcl-2 family members Bax, Puma and Noxa in hepatocytes infected with HBx-negative constructs. Playing an important role in viral replication, transcription and signal transduction HBx might be the viral protein responsible for the induction of cellular stress and DNA damage. Lacking central features essential for the viral life cycle, HBx-negative constructs induce only minor cellular stress and possibly DNA damage which is comparable with adenoviral GFP infection.

Our observations suggest that not the missing HBx itself, but rather the lack of p53 stabilization in hepatocytes infected with HBx-negative constructs is responsible for the reduced activation of CD95, Bax, Puma and Noxa. Hence, we hypothesise that HBx in not directly involved in the activation of death receptor-dependent cell death but that it is responsible for the induction of cellular stress and DNA damage upon HBV infection and the following activation of the DNA damage response and the extrinsic as well as intrinsic apoptosis signalling pathway which are finally leading to the elimination of HBV-infected hepatocytes. The direct role of HBx in the induction of cellular stress and DNA damage still needs to be investigated. Thus, further studies addressing the mechanism by which HBV induces the activation of the DDR are important aspects to be investigated in the future.

4.5 HBV infection, p53, CD95 and CD95L

The expression of a functional CD95 receptor and, therefore, the possibility to activate the major apoptosis signalling pathway involved in the elimination of hepatocytes infected with HBV also indicates a link between infected cells and the immune system. Cytotoxic CD8$^+$ T cells, able to secret the CD95 receptor ligand, CD95L, are said to be the main effector cells involved in the elimination of HBV-infected hepatocytes. We have clearly demonstrated in the present study that the CD95 receptor, up-regulated upon HBV infection, is functional. It was shown that CD95-mediated elimination of HBV-infected hepatocytes is dependent on an intact p53 status and the further molecular mechanisms regulating the activation of the extrinsic and intrinsic apoptosis signalling pathway have been described in detail.

In the present study we provide evidence that HBV-infected hepatocytes are indeed sensitive towards CD95-mediated apoptosis induced by CD95L secreted by lymphocytes. Beside the increased induction of apoptosis observed after CD95L addition (c.f. figure 3.8), experiments with multicellular tumour spheroids visualized the effect of CD95L on HBV-infected hepatocytes (c.f. figure 3.9 and 3.10). Multicellular tumour spheroids (MCTS) are a well-established 3-D *in vitro* system simulating the pathophysiological *in vivo* situation in tumour microregions. Spheroids are a commonly used system to study tumour-immune cell interactions. Spheroids generated with Hep3B cells were infected with HBV and/or treated with CD95L containing supernatant of J16 Jurkat cells. To boost the effect of HBV, cells were additionally co-infected with adenoviral p53. The importance of the CD95-CD95L system for the elimination of hepatocytes was already demonstrated in uninfected cells. Even though there were no infectious particles available in the medium which could trigger DNA damage and the following up-regulation of CD95, the addition of CD95L reduced the migration radius of the spheroids compared to untreated spheroids. This observation confirmed the conclusion discussed in several papers that CD95 is constitutively expressed in the liver leading to a high sensitivity towards CD95-mediated apoptosis of hepatocytes (Galle, Hofmann et al. 1995; Schilling, Schleithoff et al. 2009). While HBV infection alone merely led to a decreased migration of hepatocytes out of the spheroid, HBV infection combined with the CD95L-treatment resulted in marginal disaggregation of the spheroid and almost no cell migration. We, therefore, hypothesise that the addition of CD95L overcomes the

resistance of p53-deficient cells towards CD95-mediated apoptosis. As already mentioned above, upon severe DNA damage the p53 family member p73 seems to undertake the role of p53. Moreover, Müller et al. have been shown that TAp73 is indeed able to induce CD95 gene transactivation (Müller, Schilling et al. 2005). Based on the fact that p73 leads to the up-regulation of CD95 in p53-deficient hepatocytes, the disaggregation of the spheroid can be explained. The addition of exogenous Adp53 boosted the disaggregation effect and resulted in increased apoptosis of infected hepatocytes confirming the outstanding role of p53 in the CD95-mediated apoptosis of HBV-infected hepatocytes (c.f. figure 3.9).

The role of the other p53 family members, p63 and p73, in the elimination of HBV-infected hepatocytes and their ability to combat p53 alterations in general has been studied by two MD students, Long-An Sun and Annabel Langhein, in our laboratory (unpublished data).

4.6 AP-1-dependent CD95L transactivation, transcription and expression

Another transcription factor which is phosphorylated upon HBV infection is the stress activated c-Jun N-terminal kinase (JNK). In the present study the role of JNK in the transactivation of the CD95L gene in HBV-infected hepatocytes could be clarified. CD95L expression is generally restricted to a few cell types, e.g. T cells and NK cells. However, it has been reported recently, that hepatocytes are not only sensitive towards CD95-mediated apoptosis but also able to express CD95L by themselves and are therefore able to eliminate other cells via CD95L-CD95-mediated cytotoxicity. Müller et al. previously identified a novel AP-1 element in the CD95L promoter to be responsible for the induction of apoptosis upon treatment with chemotherapy in hepatoma cells (Eichhorst, Müller et al. 2000). c-Jun, a component of the transcription factor AP-1, was identified as an important protein involved in CD95L transactivation, which could be confirmed in the present study by the use of a JNK inhibitor and the following reduction in CD95L transactivation in bleomycin-treated hepatocytes (c.f. figure 3.14).

HBV infection resulted not only in the phosphorylation of JNK, the upstream regulator of c-Jun, and, therefore, the transcription factor AP-1; it also triggered the transactivation, transcription and expression of CD95L.

Due to the use of (i) four distinct CD95L reporter constructs, differing in the length of the upstream part of the promoter and thus possessing or lacking specific binding sites, inhibitors or enhancers, (ii) the use of a JNK inhibitor as well as (iii) 5' UTR AP-1 binding site deletion constructs, JNK could be identified as the prevalent protein involved in CD95L transactivation in HBV-infected hepatocytes.

Since the use of the four different reporter constructs did not favour a binding site upstream the promoter, a potential binding site in the 5' UTR of the CD95L gene was tested for its role in CD95L transactivation. While there were only minor differences in the transactivation fold increase of the different promoter constructs, the use of a JNK inhibitor (JNK inhibitor II) led to a significant decrease in the transactivation of CD95L pointing to the involvement of an AP-1 binding site in transactivation of the CD95L gene (c.f. figure 3.17 and 3.18). Taking the fact into account that the use of a JNK II inhibitor resulted in the inhibition of CD95L transactivation and that HBV infection led to the phosphorylation of JNK, we postulate that CD95L transactivation in HBV-infected hepatocytes is triggered by Thr[183]/ Tyr[185] phosphorylation of JNK and the subsequent activation of AP-1. Deletion of the 5' UTR AP-1 binding site located between +84 and +91 in the CD95L reporter gene constructs resulted in a decreased CD95L transactivation similar to the treatment with the JNK inhibitor. Thus, the results obtained here suggest that the AP-1 binding site in the 5' UTR of the CD95L gene is the major binding site responsible for the transactivation of the CD95L gene in HBV-infected hepatocytes (c.f. figure 3.20).

HBV infection did not only lead to the transactivation of the CD95L gene, it also resulted in the transcription and expression of CD95L. The human CD95L exists in a soluble (sCD95L) and a membrane-bound form (mCD95L), whereby the soluble form is generated from the membrane-bound form through cleavage by metalloproteinase on the cell surface (Strauss, Osen et al. 2007). In contrast to mCD95L which is triggering CD95-mediated apoptosis by the formation of trimers or even higher molecular structures very efficiently, the role of sCD95L in apoptosis induction is still controversial (Strauss, Osen et al. 2007). Nevertheless, the results obtained in the present study show that sCD95L undoubtedly induces apoptosis (c.f. figure 3.8, treatment of HBV-infected hepatocytes with SNJ16). While the amount of sCD95L already increased slightly upon infection with AdHBV X-, and even more upon infection with HBx containing constructs, the expression of mCD95L showed the opposite pattern with AdHBV wt-harbouring cells having the least amount of mCD95L

as detected by ELISA. Similar pattern in CD95L expression was seen in cells treated with bleomycin. Upon bleomycin stimulation sCD95L expression increased and mCD95L expression dropped (c.f. figure 3.15). On the contrary, CD95L FACS which only detects membrane-bound CD95L first showed an increase (48 h p.i.) in mCD95L in cells infected with the HBx-containing constructs, before the amount of mCD95L fell back to the AdGFP level at 72 h p.i. Therefore, it is postulated that the majority of mCD95L is cleaved off from the cell membrane by metalloproteinases at 48 h to 72 h after HBV infection. The cleavage of mCD95L by metalloproteinases is a well feasible mechanism resulting in an increased amount of sCD95L which is able to trigger the activation of the CD95L-CD95-mediated apoptosis signalling pathway in neighbouring hepatocytes. The secretion of sCD95L enables the infected cells to kill and to be killed, nevertheless, there are indications in the literature that mCD95L is also playing an important role in the induction of apoptosis (Barnhart, Pietras et al. 2005). Therefore, further investigations on the expression of sCD95L and mCD95L upon HBV infection could provide valuable insights into the so far uncovered details of apoptosis signalling and they definitely deserve further attention.

Knowing that HBV infection triggers CD95L expression via the activation of JNK and the subsequent AP-1-dependent activation of CD95L transcription the question regarding the upstream mechanism of JNK phosphorylation emerged. The tumour necrosis factor -alpha (TNF-α) was described as a possible inducer of JNK activation (Lin and Dibling 2002; Liu and Lin 2005). Indeed, additional treatment of HBV-infected hepatocytes with recombinant TNF-α led to a significant increase in CD95L transactivation suggesting a potential role of TNF-α in the induction of CD95L and maybe JNK phosphorylation. Inhibition of TNF-α with Enbrel, a TNF-α inhibitor, however, did not result in a decreased CD95L transactivation (c.f. figure 3.21). Regarding these results we postulate that JNK phosphorylation and the subsequent transactivation of CD95L in HBV-infected hepatocytes are independent of TNF-α. Beside TNF-α, reactive oxygen species (ROS) have been shown to be crucial for activation-induced CD95L expression in T cells. The ROS-inducible transcription factors AP-1 and NF-κB were found to be responsible for final CD95L expression and CD95-dependent apoptosis in T cells (Kaminski, Kiessling et al. 2007; Kiessling, Linke et al. 2010). The role of ROS in the HBV-induced inflammation-cirrhosis-cancer axis is well defined. Oxidative stress induced either by the virus itself or by the

immune response of the host, is suggested to be an important pathological mechanism in chronic liver diseases (Lee, Hwang et al. 2004). The role of HBx-induced ROS in hepatocellular carcinogenesis was described previously by Ha et al. indicating a ROS-mediated dysregulation of PTEN/Akt pathway (Ha and Yu 2010). The HBx-mediated loss of MOMP is not only linked to cell death, but also to oxidative liver injury which is induced by the release of ROS. Alteration of the mitochondrial membrane potential results in the activation of several response stimuli amongst others the production of ROS. Furthermore, HBx was identified to be the crucial protein needed for ROS production and the subsequent activation of the transcription factors NF-κB and STAT-3 (Waris, Huh et al. 2001; Lim, Kwon et al. 2010). The correlation between ROS, JNK and AP-1 in the cell death of hepatocytes has been illustrated recently (Czaja 2003). In contrast to Melino et al. (Melino, Bernassola et al. 2000), who showed that the reactive oxygen species-nitric oxide (NO) inhibits the AP-1 dependent CD95L transactivation in Jurkat T cells, our data provides evidence for the ROS-dependent CD95L transactivation in hepatocytes. Blocking of ROS with the reactive oxygen species inhibitor N-acetyl L-cysteine (NAC) significantly decreased the CD95L transactivation in HepG2 cells upon bleomycin treatment or HBV infection. This is in accordance with Kaminski et al. (Kaminski, Kiessling et al. 2007), who demonstrated that ROS enhance NF-κB and AP-1 activation and therefore, the subsequent NF-κB and AP-1-dependent up-regulation of CD95L transactivation in T cells. Since ROS-mediated cell death of hepatocytes is associated with an overexpression of JNK/AP-1 (Czaja 2003) and since we observed that ROS inhibition results in the decrease of CD95L transactivation in HBV-infected hepatocytes, we hypothesise that HBV-induced phosphorylation of JNK and the subsequent CD95L transactivation are dependent on ROS.

Similar to the results obtained for CD95 regulation, infection with the HBx knock-out adenoviral HBV construct, AdHBV X-, resulted in a minor transactivation, transcription and expression of CD95L. As HBx was identified as the crucial protein needed for ROS production (Lim, Kwon et al. 2010) and as we identified ROS to be essential for CD95L transactivation in HBV-infected hepatocytes, we are able to explain the decreased CD95L transactivation and expression in AdHBV X-infected hepatocytes. This is in accordance with Kasibhatla, Shin, Lee and Yoo who found that HBx induces CD95L activity through an Egr (early growth response protein) binding site by interacting and increasing the transcriptional activity of Egr 2 and

Egr 3 and, therefore, inducing its transactivational function (Kasibhatla, Genestier et al. 1999; Shin, Shin et al. 1999; Lee, Kang et al. 2001; Yoo and Lee 2004). While the authors' results regarding the stimulatory role of HBx could be confirmed in the present study, the identity of the transcription factor responsible for CD95L transactivation was contradictory. Instead of Egr, AP-1 binding to its 5' UTR binding site was identified as the major event responsible for CD95L transactivation.

The results obtained here indicate that HBV infection leads to the transactivation, transcription and expression of sCD95L, proposing a new mechanism for the elimination of HBV-infected hepatocytes independently of cytotoxic T cells. However, sCD95L secretion of HBV-infected hepatocytes and, therefore, the self-killing of hepatocytes do not lessen the important role of cytotoxic T cells in the elimination of virus-infected cells. So far, it is indisputable that cytotoxic T cells are the main effector cells involved in virus elimination by a polyclonal, vigorous and multi-specific response. This has been clearly shown in transgenic mice, chimpanzees and last but not least in patients infected acute or chronically with HBV (Chisari 1997; Thimme, Wieland et al. 2003). In conclusion and of clinical importance our findings propose an additional new pathway for the elimination of HBV-infected hepatocytes.

In summary, upon the activation of the DNA damage pathway, p53 promotes cell death via the extrinsic and intrinsic apoptosis signalling pathway in HBV-infected hepatocytes. Stabilization of p53 is achieved by ATM/ATR-dependent serine 15 phosphorylation and JNK-mediated abolishment of p53-Mdm2 interaction. We could show that the p53 target genes CD95 and Bax are transactivated by p53 resulting in an up-regulation of the CD95 receptor, which is crucial for the elimination of HBV-infected hepatocytes as well as the activation of Bax-mediated cytochrome c release. The tumour suppressor p53 and its target CD95 were identified as essential proteins for the elimination of HBV-infected hepatocytes. The particular role of CD95 was confirmed in a previously performed in vivo experiment in our laboratory. The results of the in vitro and in vivo experiments highlight the important roles of p53 and CD95, without both, neither the induction of apoptosis nor the onset of a proper immune response was possible. Several in vitro experiments verified the responsiveness of HBV-infected cells towards CD95-mediated apoptosis and provided further evidence for the molecular mechanisms involved in the elimination of HBV-infected hepatocytes. The present study furthermore suggests a new mechanism for the elimination of HBV-infected hepatocytes through the secretion of sCD95L by infected hepatocytes themselves. The 5' UTR AP-1 element was identified as the crucial binding site for CD95L transactivation in HBV-infected hepatocytes.

In conclusion, the important role of an intact p53 status for the elimination of HBV-infected hepatocytes, the subsequent activation of the apoptosis signalling pathways and the ability of hepatocytes to secret CD95L may be an underlying mechanism for the diverse outcomes of an HBV infection (HBV clearance vs. chronicity). However, it should be kept in mind and this has also be shown in detail in the present work that even though p53 and CD95 could be identified as crucial proteins involved in the elimination of HBV-infected hepatocytes, the elimination of HBV-infected cells is regulated by a large and connected network of proteins and pathways working together to obtain a sufficient virus eradication.

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6. Appendix

6.1 Abbreviations

aa	amino acid	MLH1	MutL protein homologue 1
ADCC	antibody-dependent cell-mediated cytotoxicity	MOI	multiplicity of infection
ALT	alanine amino transferase	MOMP	mitochondrial outer membrane potential
APC	antigen-presenting cell	MRN complex	MRE11-Rad50-NBS1
AST	aspartate transaminase	mRNA	messenger RNA
ATM	ataxia-teleangiectasia mutated	NK cells	natural killer cells
ATR	ataxia-teleangiectasia and Rad3 related	Na	sodium
Bax	Bcl-2-associated X	NAC	N-acetyl L-cysteine
BH3	Bcl-2-homology domain-3	O_2	oxygen
BS	binding site	O/N	over night
cccDNA	covalently closed circular DNA	ORF	open reading frame
℃	degree Celsius	PAR	poly(ADP-ribose)-polymers
CHK1/2	checkpoint kinase	PARP-1	poly(ADP-ribose)-polymerase 1
c.f.	confer, compare	PBS	phosphate buffered saline
CMV	cytomegalovirus immediate/early gene promoter	PCR	polymerase chain reaction
Δ	delta	p.i.	post infection
DDR	DNA damage response	P/I	PMA/Ionomycin
DISC	death-inducing signalling complex	PHH	primary human hepatocytes
DNA	deoxyribonucleic acid	pgRNA	pregenomic RNA
dsDNA	double-stranded DNA	PMSF	phenylmethylsulfonylfluorid
DTT	dithiothreitol	PRMT5	protein arginine methyltransferase 5
ELISA	enzyme-linked immunosorbent assay	REs	response elements
FACS	fluorescent-activated cell sorting	rcDNA	relaxed circular viral DNA
FCS	fetal calf serum	rpm	revolutions per minute
FADD	Fas-associated death domain	ROS	reactive oxygen species
HBV	hepatitis B virus	RT	room temperature
HEPES	4-(2-hydroxyethyl)-1piperazineethanesulfonic acid	sCD95L	soluble CD95L
HCC	hepatocellular carcinoma	SDS	sodium dodecyl sulphate
HSCs	hepatic stellate cells	sgRNA	subgenomic RNA
H_2O	water	SMYD2	SET and MYND domain-containing 2
IU	infectious units	SVPs	subviral enveloped particles
IFN	interferon	TLR	toll-like receptor
JNK	Jun N-terminal kinase	TNF	tumour necrosis factor
Kat5	K (lysine) acetyltransferase 5	TNFR	tumour necrosis factor receptor
LSECs	liver sinusoidal endothelial cells	U	units
miRNA	microRNA	UTR	untranslated region
MCTS	multicellular tumour spheroid	UV	ultraviolet light
mCD95L	Membrane-bound CD95L	wt	wild-type
MEKK1	mitogen-activated protein kinase kinase kinase 1	53BP1	53 binding protein 1
MKK4	mitogen-activated protein kinase kinase 4		

6.2 Size units and physical scales

A	ampere	**min**	minutes
°C	degree Celsius	**rpm**	revolutions per minute
g	gram	**s**	seconds
h	hours	**U**	units
l	litre	**V**	volt
M	molar (mol/l)	**W**	watt

6.3 Prefix of scale units

M	mega 10^6	**µ**	micro 10^{-6}
k	kilo 10^3	**n**	nano 10^{-9}
c	centi 10^2	**p**	pico 10^{-12}
m	milli 10^{-3}	**f**	femto 10^{-15}

6.4 Three letter code of amino acids

Ala	alanine	**Met**	methionine
Cys	cysteine	**Asn**	asparagine
Asp	aspartic acid	**Gln**	glutamine
Glu	glutamic acid	**Arg**	arginine
Phe	phenylalanine	**Ser**	serine
Gly	glycine	**Thr**	threonine
His	histidine	**Val**	valine
Ile	isoleucine	**Trp**	trypthophane
Lys	lysine	**Tyr**	tyrosine
Leu	leucine		

6.5 Plasmid gene cards

(a)

CD95 Ligand Luc Plasmids
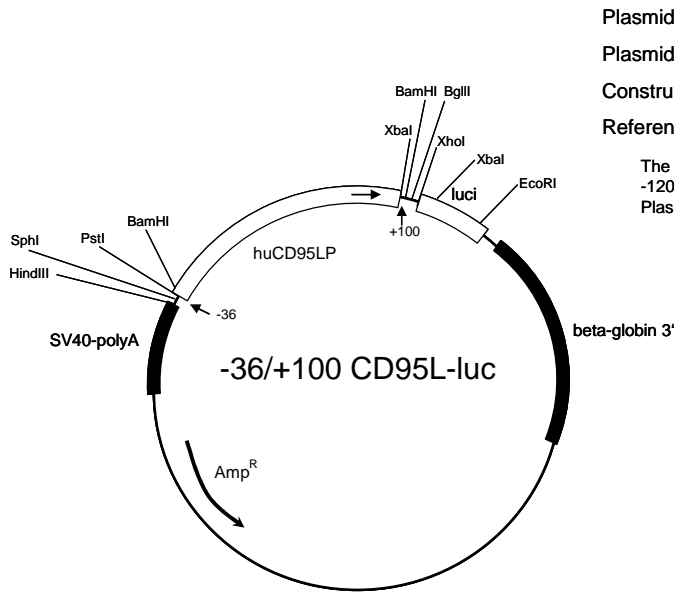
Plasmid name: CD95L -36/+100 Luc

Plasmid size: Vector 5.5kb, insert -36/+100bp

Constructed by: Min Li-Weber

References: Mol. Cell. Bio., 2000, 20:7826-7837

The plasmids containing -220/+100, -537+100 and -1204/+100 were generated in the same way. Plasmid cards not shown here.

(b)

CATCAATGTATCTTATCATGTCTGGATCTCG*AAGCTT*AACTC
-36
TATAAGAGAGATCCAGCT
TGCCTCCTCTTGAGCAGTCAGCAACAGGGTCCCGTCCTTGACACCTCAGCCTCTACA
GGACTGAGAAGAAGTAAAACCGTTTGCTGGGGCTGGCCTGACTCACCAGCTGCCTC
TAGAG GAT*CTCGAG*ATCCATTCCGGTACTGTTGGTAAAATGGAAGACGCCAAAAACAT
AAAGAAAGGCCCGGCGCCATTCTATCCTCTAGAGGATGGAA CCGCTGGAGA

HindIII/ XhoI
Flanking Plasmid Sequence
TATA Box
AP-1 Binding Site

-220
*AAGCTT*CAGCAACTGAGGCCTTGA**AGGCTGTTATCAGAAAATTGTGGGCGGAAACTTC**
CAGGGGTTTGCTCTGAGCTTCTTGAGGCTTCTCAGCTTCAGCTGCAAAGTGAGTGGGT
GTTTCTTTGAGAAGCAGAATCAGAGAGAGAGAGATAGAGAAAGAGAAAGACAGAGGT
GTTTCCCTTAGCTATGGAAACTCTATAAGAGAGATCCAGCTTGCCTCCTCTTGAGCAGT
CAGCAACAGGGTCCCGTCCTTGACACCTCTACAGGACTGAGAAGAAGTAAA
ACCGTTTGCTGGGGCTGGCCTGACTCACCAGCTGCCTCTAGAGGATCCAGAT*CTCGAG*

-537
*AAGCTC*ATGGTCTCTCCCCTCAGAGCCAT
TTTCAGTAAAATTTTTATAGTTTAAAAAATACGGTTCTGAACAATTTTTGTTAGAGTTAT
TTTGGGATTTTTAAATAGTTTTTTGGTTGTGTATATGGTAGAATTCTTAAAATTATACAA
TTATAATGTATAAAAAAGCATGCAATTATAATTCATAAAATTATAGCCCCACTGACCATT
CTCCTGTAGCTGGGAGCAGTTCACACTAACAGGGCTATACCCCCATGCTGACCTGCTC
TGCAGGATCCCAGGAAGGTGAGCATAGCCTACTAACCTGTTTGGGTAGCACAGCGACA
GCAACTGAGGCCTTGAAGGCTGTTATCAGAAAATTGTGGGCGGAAACTTCCAGGGGTT
TGCTCTGAGCTTCTTGAGGCTTCTCAGCTTCAGCTGCAAAGTGAGTGGGTGTTTCTTTG
AGAAGCAGAATCAGAGAGAGAGAGATAGAGAAAGAGAAAGACAGAGGTGTTTCCCTT
AGCTATGGAAACTC**TATAAGA**GAGATCCAGCTTGCCTCCTCTTGAGCAGTCAGCAACA
GGGTCCCGTCCTTGACACCTCAGCCTCTACAGGACTGAGAAGAAGTAAAACCGTTTGC
TGGGGCTGGCC**TGACTCA**CCAGCTGCCTCTAGAGGA*TCTCGAG*

-1204
*AAGCTTC*GAGGGTGTCTGATATTCTGATATTTCAAAACAGAATAGAAATATGTATTTTAA
TGTGTATTTAATATATATATTTTAAAATATTTTTAATGTTGATACTAAAAGTGGTCAGTTG
GGCTCAGTGCAGTGGCTCATGCCTGTAATTCCAGCACTTTGGGAAACTGAGGCAGGAG
GATGTCTTGAAGCTAGGAGTTTGAGACAAGCCTGGGCAAC{...}
AGGTGTTTCCCTTAGCTATGGAAACTC**TATAAGA**GAGATCCAGCTTGCCTCCTCTTGAG
CAGTCAGCAACAGGGTCCCGTCCTTGACACCTCAGCCTCTACAGGACTGAGAAGAAGT
AAAACCGTTTGCTGGGGCTGGCC**TGACTCA**CCAGCTGCCTCTAGAGGAT*CTCGAG*

Figure 6.1 Overview of the CD95L reporter plasmids. (a) Map of the -36/+100 CD95L-luc plasmid used for CD95L reporter assays. Shown are the main restriction sites, the antibiotic resistance and the insert. The plasmid was constructed by Min Li-Weber (DKFZ) who generously provided the different plasmids. **(b)** Sequences of the different reporter constructs. The flanking plasmid sequence is shown in blue (only shown for the -36/+100 construct). The used restriction sites (HindIII and XhoI) are shown in turquoise, the AP-1 binding site in purple and the TATA Box in red.

(a)

CD95 Ligand Luc Plasmids (AP-1 Deletion)

Plasmid name: CD95L -36/+81 Luc

Plasmid size: Vector 5.5kb, insert -36/+81bp

Constructed by: Natalie Joschko (original plasmid provided by Min Li-Weber (DKFZ)

References: Mol. Cell. Bio., 2000, 20:7826-7837

The plasmids containing -220/+100, -537+100 and -1204/+100 were generated in the same way. Plasmid cards not shown here.
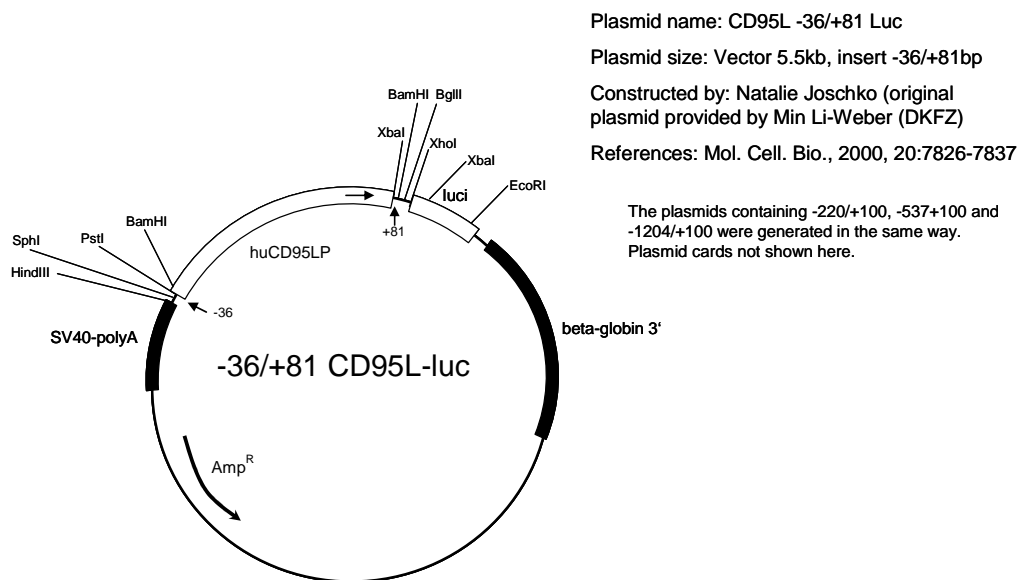
(b)

-36
*AAGCTT*AACTC
TATAAGAGAGATCCAGCTTGCCTCCTCTTGAGCAGTCAGCAACAGGGTCCCGTCCTT
GACACCTCAGCCTCTACAGGACTGAGAAGAAGTAAAACCGTTTGCTGGGGCTGGCC
CTCGAG

HindIII/ XhoI
Flanking Plasmid Sequence
TATA Box

-220
*AAGCTT*CAGCAACTGAGGCCTTGA**AGGCTGTTATCAGAAAATTGTGGGCGGAAACTTC**
CAGGGGTTTGCTCTGAGCTTCTTGAGGCTTCTCAGCTTCAGCTGCAAAGTGAGTGGGT
GTTTCTTTGAGAAGCAGAATCAGAGAGAGAGAGATAGAGAAAGAGAAAGACAGAGGT
GTTTCCCTTAGCTATGGAAACTC**TATAAGA****GAGATCCAGCTTGCCTCCTCTTGAGCAGT**
CAGCAACAGGGTCCCGTCCTTGACACCTCAGCCTCTACAGGACTGAGAAGAAGTAAA
ACCGTTTGCTGGGGCTGGCC*CTCGAG*

-537
*AAGCTC*ATGGTCTCTCCCCTCAGAGCCAT
TTTCAGTAAAATTTTTATAGTTTAAAAAATACGGTTCTGAACAATTTTTGTTAGAGTTAT
TTTGGGATTTTTAAATAGTTTTTTGGTTGTGTATATGGTAGAATTCTTAAAATTATACAA
TTATAATGTATAAAAAAGCATGCAATTATAATTCATAAAATTATAGCCCCACTGACCATT
CTCCTGTAGCTGGGAGCAGTTCACACTAACAGGGCTATACCCCCATGCTGACCTGCTC
TGCAGGATCCCAGGAAGGTGAGCATAGCCTACTAACCTGTTTGGGTAGCACAGCGACA
GCAACTGAGGCCTTGAAGGCTGTTATCAGAAAATTGTGGGCGGAAACTTCCAGGGGTT
TGCTCTGAGCTTCTTGAGGCTTCTCAGCTTCAGCTGCAAAGTGAGTGGGTGTTTCTTTG
AGAAGCAGAATCAGAGAGAGAGAGATAGAGAAAGAGAAAGACAGAGGTGTTTCCCTT
AGCTATGGAAACTC**TATAAGA**GAGATCCAGCTTGCCTCCTCTTGAGCAGTCAGCAACA
GGGTCCCGTCCTTGACACCTCAGCCTCTACAGGACTGAGAAGAAGTAAAACCGTTTGC
TGGGGCTGGCC*CTCGAG*

-1204
*AAGCTT*CGAGGGTGTCTGATATTCTGATATTTCAAAACAGAATAGAAATATGTATTTTAA
TGTGTATTTAATATATATATTTTAAAATATTTTTAATGTTGATACTAAAAGTGGTCAGTTG
GGCTCAGTGCAGTGGCTCATGCCTGTAATTCCAGCACTTTGGGAAACTGAGGCAGGAG
GATGTCTTGAAGCTAGGAGTTTGAGACAAGCCTGGGCAAC{...}
AGGTGTTTCCCTTAGCTATGGAAACTC**TATAAGA**GAGATCCAGCTTGCCTCCTCTTGAG
CAGTCAGCAACAGGGTCCCGTCCTTGACACCTCAGCCTCTACAGGACTGAGAAGAAGT
AAAACCGTTTGCTGGGGCTGGCC*CTCGAG*

Figure 6.2 Overview of the CD95L reporter plasmids (AP-1 Deletion). (a) Map of the -36/+81 CD95L-luc plasmid used for CD95L reporter assays. Shown are the main restriction sites, the antibiotic resistance and the insert. **(b)** Sequences of the different reporter constructs. The used restriction sites (HindIII and XhoI) are shown in turquoise, the AP-1 binding site in purple and the TATA Box in red.

6.6 List of tables

6.7 List of figures

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Parts of the present work have been presented at conferences:

Elimination Hepatitis B Virus (HBV)-infizierter Hepatozyten durch CD95-mediierte Apoptose. **Joschko N.**, Rademacher A., Langhein A., Sun L.-A., Koch A., Stremmel W., Müller M. Z. **Gastroenterol. 2009** (9); 47: P338 (DGVS 2009, Hamburg, Germany); Poster presentation

p53 „gain-of-function" mutants inhibit apoptosis of HBV-infected hepatocytes. **Joschko N.**, Rademacher A., Langhein A., Sun L-A., Steinebrunner N., Koch A., Stremmel W., Müller M. **International PhD Student Cancer Conference 2010** (Milan, Italy); Poster presentation

Elimination Hepatitis B Virus (HBV)-infizierter Hepatozyten durch die Induktion mitochondrialer Apoptose-Signalwege. **Joschko N**, Steinebrunner N., Protzer U. Stremmel W., Müller M. Z **Gastroenterol 2010** (8); 48: P601 (DGVS 2010, Stuttgart, Germany); Poster presentation

Interaction of Hepatitis B virus (HBV) with apoptosis signalling pathways is involved in viral clearance. Steinebrunner N., **Joschko N.,** Stremmel W., Müller M. **18th Euroconference on Apoptosis (ECDO) 2010** (Gent, Belgium); Poster presentation

The p53 family is involved in the elimination of Hepatitis B virus (HBV)-infected hepatocytes. Sun, LA; Steinebrunner, N; **Joschko, N**; Langhein, A; Stremmel, W; Protzer, U; Müller-Schilling, M. Z **Gastroenterol 2011**; 49: P4_45 (GASL 2011, Regensburg, Germany); Poster presentation

Hepatitis B Virus infection triggers the activation of p53 downstream target genes in primary human hepatocytes. **Joschko N**., Steinebrunner N., Stremmel W., Müller M. **19th Euroconference on Apoptosis (ECDO) 2011** (Stockholm, Sweden); Poster presentation

Multicellular tumour spheroids – A model system for investigation of CD95Ligand-induced apoptosis in HBV-infected hepatocytes. **Joschko N**., Lovas A., Steinebrunner N., Sun L.-A., Stremmel W., Müller M. **The Liver Meeting® 2011 (AASLD)** (San Francisco, USA); Poster presentation

Hepatitis B Virus Infection triggers AP-1 dependent CD95Ligand expression in HepG2 cells. **Joschko N**., Krammer P.H., Stremmel W., Müller M. **(GASL 2012, Hamburg, Germany)**; Poster presentation

Manuscripts in preparation:

The role of the CD95 system in the elimination of Hepatitis B Virus infected hepatocytes: implication for viral clearance. **N. Joschko,** T. Schilling, L. Sun, A. Langhein, A. Rademacher, U. Protzer, P.H. Krammer, M. Müller

Hepatitis B Virus triggers AP-1 dependent CD95Ligand expression in infected liver cells. **N. Joschko,** M. Li-Weber, M. Meinhard, U. Protzer, P.H. Krammer, M. Müller

Elimination of HBV-infected hepatocytes is mediated via the intrinsic apoptosis signalling pathway. **N. Joschko,** A. Rademacher, M. Müller